IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Axel Kallies, et al.	**Examiner:**	Ileana Popa
Serial No:	10/589,321	**Art Unit:**	1633
Filed:	November 22, 2006	**Docket:**	20155
For:	MODIFIED CELLS AND METHODS OF USING SAME		

Confirmation No: 6068

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, Dr. Stephen Nutt hereby declare as follows:

1. I hold a Bachelor of Science (B.S.) Degree in Biology and a Doctorate Degree in Immunology. I have conducted research in immunology since 1990. I am currently employed by The Walter and Eliza Hall Institute of Medical Research, and am involved in research relating to the control of the immune response. My research focuses particularly on the function of a small group of master regulatory transcription factors on the terminal differentiation of B and T lymphocytes. I have authored 80 publications. A true and correct copy of my curriculum vitae is attached hereto as **Exhibit 1**.

2. I am one of the co-inventors named in the above-identified application (hereinafter referred to as the '321 application). I have reviewed the '321 application and am familiar with the subject matter disclosed and claimed therein. I have also read the Office Action

dated April 1, 2009. I have been asked to review and comment on the prior art cited in the Office Action against the '321 application.

3. It is my understanding that the Examiner holds the opinion that it would have been obvious for those skilled in the art to follow the teachings of Glimcher (U.S. Publication No. US 2002/0059652) and Schaffer (*Immunity, 17*(1): 51-62, 2002), and substitute Blimp-1 for XBP-1. The Examiner's basic premise for her opinion is that these references teach that XBP-1 acts downstream of Blimp-1, and that Blimp-1 is the master regulator of terminal differentiation, wherein Blimp acts by allowing the expression of specific transcription factors such as XBP-1.

4. As a scientist skilled in the art of transcriptional regulation and terminal differentiation of lymphocytes, it is my opinion that based on a careful reading of Glimcher, Shaffer and other references available at the relevant time, it was not possible to conclude that XBP-1 acts specifically downstream of Blimp-1. In fact, XBP-1 had been shown to be expressed both upstream and downstream of Blimp-1.

a. While it is my experience that XBP-1 is highly expressed in plasma cells, it was well known in the art prior to the filing of the '321 application that that XBP-1 is highly expressed in a number of other cell types and is highly induced by the cellular stress response system. See, for example, Glimcher, paragraph [0003] states: "*this transcription factor is expressed ubiquitously in adults but is mainly found in exocrine glands and bone precursors in the embryonic mouse...*". This is in direct contrast to Blimp-1 which is not ubiquitously expressed in the adult, but has widespread expression in the embryonic mouse (Chang and Calame, *Mechanisms of Development, 117*: 305-309, 2002, attached hereto as **Exhibit 2**).

b. Shaffer states on page 55, 2nd paragraph:

> "*We confirmed the induction of XBP-1 mRNA in RAJI cells expressing Blimp-1-ERD using a quantitative RT-PCR assay (Figure 3A) and found the induction to be in the same range as detected by microarray analysis (1.7-fold on average). This XBP-1 level was substantially lower than the XBP-1 level in a myeloma cell line (Figure 3A), indicating that Blimp-1 alone is insufficient to achieve the high XBP-1 expression characteristic of plasma cells.*"

This confirms that Blimp-1 is not equivalent to XBP-1 as a marker of B cell terminal differentiation. In addition, the Shaffer study did not address whether Blimp-1 is upstream of XBP-1 in the physiological setting of primary B cells. The study instead used only human transformed cell lines to assay XBP-1 expression. Transformed B cell lines contain many chromosomal abnormalities and have lost the ability to undergo physiologically relevant terminal differentiation. Shaffer did use primary mouse B cells in Figure 4, although in this case XBP-1 was not assayed and thus no conclusion can be made on XBP-1 regulation. Hence, it is my opinion that those skilled in the art would not have drawn the conclusion, based on the results from using transformed B cells alone, that XBP-1 acts downstream of Blimp-1.

c. Reimold *et al.* (*Nature, 412*(6844) :300-307, 2001, attached hereto as **Exhibit 3**) is a publication from the Glimcher group. Figure 2 of the reference is entitled *"The induction of XBP-1 is upstream and downstream of signals that drive plasma cell differentiation"*. The data in Figure 2 and the corresponding text under the heading "Upstream and downstream roles of XBP-1" on page 301 clearly show that XBP-1 can be induced and function in B cells prior to the initiation of terminal differentiation, and that XBP-1 can also function during the terminal differentiation process. In contrast, the '321 application has shown that Blimp-1 only functions downstream of the initiation of terminal differentiation (see page 66, line 29 to page 67, line 4 of the specification).

d. Iwakoshi *et al.* (*Nature Immunology, 4*(4):321-329, 2003 attached hereto as **Exhibit 4**) show in Figure 3b that at time point 0, XBP-1 was expressed, while Blimp-1 was not, indicating that XBP-1 expression was induced prior to Blimp-1. The authors also state that "*Indeed, expression of Blimp1 is normal in XBP-1-/- B cells, so it is likely that these two transcription factors have complementary rather than overlapping functions in lineage commitment*". See page 326, column 2 of the reference.

e. There was and still is no evidence that Blimp-1 directly regulates XBP-1 expression. Shaffer states that *"Blimp-1 promotes plasmacytic differentiation by extinguishing gene*

expression important for B cell receptor signaling, ..., while allowing expression of important plasma cell genes such as XBP-1" (Abstract). While Shaffer showed enforced expression of Blimp-1 in a transformed B cell line activated the plasma cell differentiation program, and induced higher expression of genes including XBP-1, Shaffer stated that the expression level "*was substantially lower than the XBP-1 level in a myeloma cell line (Figure 3A), indicating that Blimp-1 alone is insufficient to achieve high XBP-1 mRNA expression characteristics of plasma cells.*" Page 55, left column, middle paragraph. Similarly, Shapiro-Shelef *et al.* (*Immunity, 19*(4): 607-20, 2003, attached hereto as **Exhibit 5**) show that XBP-1 expression was reduced in *Blimp-1-/-* (also called *Prdm1-/-*) B cells, yet enforced expression of XBP-1 did not compensate for the absence of Blimp-1; and therefore XBP-1 is not likely to be in the same pathway as Blimp-1. In my opinion, the absence of high XBP-1 expression in Blimp-1-/- B cells was due to the impaired formation of plasma cells without Blimp-1 and hence expression of XBP-1 and any other genes highly expressed in plasma cells was lost.

5. It is my scientific opinion that prior to the filing of the '321 application, it was not possible to conclude that XBP-1 acts downstream of Blimp-1. Given the published information and the fact that XBP-1 is more widely expressed than Blimp-1, it is more reasonable to conclude that the two proteins are regulated by independent pathways.

6. Prior to the '321 application, there were no good markers in the art for fully differentiated antibody secreting cells ("ASCs" or plasma cells). It has been a unique recognition provided by the '321 application that not only substantially all ASCs express Blimp-1 in a cell population, but also no pre-plasma/ASC cells express Blimp-1. Specifically, Example 4 shows that only ASCs are Blimp-1 positive. This means that pure populations of all ASCs can be identified and purified. Similar expression profiles were also observed with terminally differentiated T cells although expression of Blimp-1 was at a lower level.

7. The specific association of the expression of Blimp in plasma cells – i.e., expression in *all* plasma cells and lack of expression in earlier stages of B and T-cell differentiation, makes Blimp an especially useful marker for identifying modulators of terminal

differentiation. In contrast, *XBP-1* is expressed upstream and downstream of signals that drive plasma cell differentiation (see paragraph 4 above). Therefore, identifying modulators of endogenous *XBP-1* expression evidently would not provide a selective method for screening for modulators of terminal differentiation. Furthermore, if only a proportion of cells endogenously express XBP-1 and only for some time, it would be more difficult to identify modulators of endogenous expression.

8. Prior to the '321 application, targeting of *Blimp-1* was considered to potentially suffer from the same problems. It was not known and could not have been predicted that *all* fully differentiated antibody secreting cells (ASCs or plasma cells) express Blimp-1. It is evident that Blimp-1's endogenous expression consistently and only in terminal differentiated haematopoietic cells, identified only in the '321 application, makes the claimed assays, and the related cells and vectors, greatly improved over the XBP-1 based methods disclosed by Glimcher. In my opinion, not only the methods of the '321 application are superior over the prior art (e.g., Glimcher), but also the results achieved by the '321 application are unexpected to those skilled in the art. Since the publication of the '321 application, the scientific community working in this area has recognized the surprising results and usefulness of this invention. To date, I have had dozens of requests for mice having a modified *blimp-1* allele with an inserted reporter gene, and have sent breeding pairs world-wide to researchers in the field.

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____ Dated: 23rd September 2009

EXHIBIT 1

BIOGRAPHICAL SKETCH: Dr Stephen L Nutt

A. Education and Training.

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Sydney	B.Sc Hons.	1985-1988	hematopoiesis
Research Institute of Molecular Pathology (IMP), and the University of Vienna, Austria	Ph D	1994-1997	immunology
Research Institute of Molecular Pathology (IMP), Vienna, Austria	Post-doc	1998	immunology
Wellcome/CRUK Gurdon Institute. University of Cambridge, UK	Post-doc	1999-2001	Developmental biology

B. Positions and Honours.

Positions and Employment

1990 – 1994 Research associate. Allelix Biopharmaceuticals Inc., Toronto, Canada.
1997 – 1998 Post-doctoral fellow. Research Institute of Molecular Pathology, Vienna, Austria.
1999 – 2000 European Molecular Biology Organization (EMBO) fellow. Wellcome/CRUK Gurdon Institute for Developmental Biology. University of Cambridge, UK.
2000 – 2001 Human Frontier Scientific Program (HFSP) fellow. Wellcome/CRUK Gurdon Institute for Developmental Biology. University of Cambridge, UK.
2001–present Senior Research Fellow. The Walter and Eliza Hall Institute, Melbourne, Australia

Other Experience and Professional Memberships

Member Australasian Society for Immunology. (Since 2002).
Scientific editor of The Walter and Eliza Hall Institute Annual Report (Since 2002).

Honours/ Awards

1994 – 1997 Vienna Biocenter / Research Institute of Molecular Pathology International Ph D studentship
1999 – 2000 EMBO long-term fellowship.
2000 – 2001 HFSP long-term fellowship.
2001 National Association of Research Fellows of Australia. Post–doctoral award winner.
2001–2006 The Walter and Eliza Hall Institute of Medical Research Metcalf Fellowship.
2005 The Walter and Eliza Hall Institute of Medical Research Burnet Prize recipient
2006-2010 Pfizer Australia Research Fellowship
2006-2010 Senior Research Fellow. National Health and Medical Research Council of Australia

C. Selected peer-reviewed publications (in chronological order).

(Publications selected from (80) peer-reviewed publications) (IF, impact factor).

1. D'Costa, K., D. Emslie, D. Metcalf, G.K. Smyth, A. Karnowski, A. Kallies, **S.L. NUTT**, and L.M. Corcoran. 2009. Blimp1 is limiting for transformation in a mouse plasmacytoma model. Blood Mar 27. [Epub ahead of print]. (IF 10.9)
2. Stehling-Sun, S., J. Dade, **S.L. NUTT**, R.P. Dekoter, and F.D. Camargo. 2009. Regulation of lymphoid versus myeloid fate 'choice' by the transcription factor Mef2c. Nat Immunol 10:289-296. (IF 26.2, 1 citation)
3. Chevier, S., C. Genton, A. Kallies, A. Karnowski, L.A. Otten, B. Malissen, M. Malissen, M. Botto, L.M. Corcoran, **S.L. NUTT**, and H. Acha-Orbea. 2009. CD93 is required for maintenance of antibody secretion and persistence of plasma cells in the bone marrow niche. PNAS 106:3895-3900. (IF 9 6)
4. **NUTT, S.L.** 2008. B-cell identity--commitment is not forever. N Engl J Med 358:82-83. (IF 52.6)
5. Pridans, C., M.L. Holmes, M. Polli, J.M. Wettenhall, A. Dakic, L.M. Corcoran, G.K. Smyth, **S.L. NUTT**. 2008. Identification of Pax5 target genes in early B cell differentiation. J Immunol 180:1719-1728. (IF 6.1, 5 citations)
6. **NUTT, S.L.**, K.A. Fairfax, and A. Kallies. 2007. BLIMP1 guides the fate of effector B and T cells. Nat Rev Immunol 7:923-927. (IF 26.3, 2 citations)
7. Kallies, A., J. Hasbold, K. Fairfax, C. Pridans, D. Emslie, B.S. McKenzie, A.M. Lew, L.M. Corcoran, P.D. Hodgkin, D.M. Tarlinton, and **S.L. NUTT**. 2007. Initiation of Plasma-Cell Differentiation Is Independent of the Transcription Factor Blimp-1. Immunity 26:555-566. (IF 19.3, 27 citations)
8. Dakic, A., L. Wu, and **S.L. NUTT**. 2007. Is PU.1 a dosage-sensitive regulator of haemopoietic lineage commitment and leukaemogenesis? Trends Immunol 28:108-114. (IF 9.5, 7 citations)
9. **Nutt, S.L.**, and B.L. Kee. 2007. The transcriptional regulation of B cell lineage commitment. Immunity 26:715-725. (IF 19.3, 37 citations)
10. Kelly, P.N., A. Dakic, J.M. Adams, **S.L. NUTT***, and A. Strasser*. 2007. Tumor growth need not be driven by rare cancer stem cells. Science 317:337. (IF 26.4, 98 citations) * equal contribution

11. Huntington, N.D., H. Puthalakath, P. Gunn, E. Naik, E.M. Michalak, M.J. Smyth, H. Tabarias, M.A. Degli-Esposti, G. Dewson, S.N. Willis, N. Motoyama, D.C. Huang, **S.L. NUTT***, D.M. Tarlinton*, and A. Strasser*. 2007. Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. Nat Immunol 8:856-863. (IF 26.2, 9 citations) * equal contribution
12. Huntington, N.D., H. Tabarias, K. Fairfax, J. Brady, Y. Hayakawa, M.A. Degli-Esposti, M.J. Smyth, D.M. Tarlinton, and **S.L. NUTT**. 2007. NK Cell Maturation and Peripheral Homeostasis Is Associated with KLRG1 Up-Regulation. J Immunol 178:4764-4770. (IF 6.1, 9 citations)
13. Park-Min, K.H., N.V. Serbina, W. Yang, X. Ma, G. Krystal, B.G. Neel, **S.L. NUTT**, X. Hu, and L.B. Ivashkiv. 2007. FcgammaRIII-Dependent Inhibition of Interferon-gamma Responses Mediates Suppressive Effects of Intravenous Immune Globulin. Immunity 26:67-78. (IF 19.3, 34 citations)
14. Kallies, A., and **S.L. NUTT**. 2007. Terminal differentiation of lymphocytes depends on Blimp-1. Curr Opin Immunol 19:156-162. (IF 9.3, 23 citations)
15. Kallies, A., E.D. Hawkins, G.T. Belz, D. Metcalf, M. Hommel, L.M. Corcoran, P.D. Hodgkin, and **S.L. NUTT**. 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. Nat Immunol 7:466-474. (IF 26.2, 38 citations)
16. Metcalf, D., A. Dakic, S. Mifsud, L. Di Rago, L. Wu, and **S. NUTT.** 2006. Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. PNAS 103:1486-1491. (IF 9.6, 23 citations)
17. Holmes, M.L., S. Carotta, L.M. Corcoran, and **S.L. NUTT**. 2006. Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment. Genes Dev 20:933-938. (IF 14.8, 10 citations)
18. Polli, M., A. Dakic, A. Light, L. Wu, D.M. Tarlinton, **S.L. NUTT**. 2005. The development of functional B lymphocytes in conditional PU.1 knock-out mice. Blood 106:2083-90. (IF 10.9, 18 citations)
19. Dakic, A., D. Metcalf, L. Di Rago, S. Mifsud, L. Wu, **S.L. NUTT**. 2005. PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. J Exp Med 201:1487-1502. (IF 15.6, 50 citations)
20. Huntington, N.D., Y. Xu, **S.L. NUTT***, and D.M. Tarlinton*. 2005. A requirement for CD45 distinguishes Ly49D-mediated cytokine and chemokine production from killing in primary NK cells. J Exp Med 201:1421-1433. (IF 15.6, 23 citations) * equal contribution
21. **NUTT, S.L.**, D. Metcalf, A. D'Amico, M. Polli, and L. Wu. 2005. Dynamic regulation of PU.1 expression in hematopoietic progenitors. J Exp Med 201:221-231. (IF 15.6, 64 citations)
22. Kallies, A., J. Hasbold, D.M. Tarlinton, W. Dietrich, L.M. Corcoran, P.D. Hodgkin, **S.L. NUTT**. 2004. Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. J Exp Med 200:967-977. (IF 15.6, 74 citations)
23. Brady, J., Y. Hayakawa, M.J. Smyth, and **S.L. NUTT**. 2004. IL-21 induces the functional maturation of murine NK cells. J Immunol 172:2048-2058. (IF 6.1, 88 citations)
24. Cook, W.D., B.J. McCaw, C.D. Herring, D.L. John, S.J. Foote, **S.L. NUTT**, and J.M. Adams. 2004. PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA-binding domain. Blood 104:3437-3444. (IF 10.9, 41 citations)
25. **NUTT, S.L.**, B. Heavey, A.G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature 401:556-562. (IF 28.8, 444 citations)
26. Rolink, A.G., **S.L. NUTT**, F. Melchers, and M. Busslinger. 1999. Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. Nature 401:603-606. (IF 28.8, 201 citations)

D. Research Support

List selected ongoing or completed (during the last three years) research projects.

Ongoing Research Support

Pfizer Australia Research Fellowship. Senior Research Fellow. 2006-2010. $220,000/year
NHMRC Senior Research Fellow. 2006-2010. Honorary
NHMRC Program Grant. The regulation of antibody: A systems approach. 2009-2013. PIs: Hodgkin P.D., Tarlinton D. M., **NUTT S.L**, Corcoran L.M. $1,534,000/year
NHMRC Project Grant. The transcriptional regulation of lymphocyte and dendritic cell development Research Grant, 2007-2009. PIs: **NUTT S.L**, Wu. L. $191,000/year
NHMRC Development Grant. Monoclonal antibodies targeting plasma cells as novel therapeutic agents and diagnostic tools. 2009. PIs: Corcoran L.M., Tarlinton D. M., **NUTT S.L**, Hodgkin P.D. $195,125.
CSL Ltd. . Monoclonal antibodies targeting plasma cells. 2006-2009. PIs: Corcoran L.M., Tarlinton D. M., **NUTT S.L**, Hodgkin P.D. ~$300,000/year.

Completed Research Support (last 3 years)

The Walter and Eliza Hall Institute Leadership Fund. Metcalf Fellowship 2001-2006. $250,000/year
NHMRC Program Grant. The regulation of antibody: A systems approach. (356202) 2005-2008. PIs: Hodgkin P.D., Tarlinton D. M., **NUTT S.L**, Corcoran L.M. $1,039,000/year
NHMRC Project Grant. Blimp-1, a master regulator of B-lymphocyte terminal differentiation (305513), 2004-2006. Corcoran LM, **NUTT S.L**, Hodgkin PD, Tarlinton DM. $151,750/ year

E. Selected Conference Presentations (2006-2009)

Gene expression and signalling in the immune system, Cold Spring Harbor, NY. USA. 04/06
3rd International Conference on Gene Regulation in Lymphocyte Development. Corfu, Greece. 10/06
37th Annual Meeting of the Scandinavian Society for Immunology. Turku, Finland, 06/07. Plenary Lecture.
Pasteur Institute. Paris, France. 06/07.
Singapore Immunology Network. A*STAR. Singapore. 06/07.
FASEB Summer Conference. Lymphocytes and the Immune System: Molecular, Cellular and Integrative Mechanisms. Tuscon, Arizona, USA, 07/07.
Australasian Society for Immunology. Sydney. 12/07. Plenary lecture.
New Direction in Leukemia Research (NDLR2008). Sunshine Coast, Qld. 04/08
Gene expression and signalling in the immune system, Cold Spring Harbor, NY. USA. 04/08
UCLA, Los Angeles, USA. 04/08
University of Miami, Florida USA. 04/08
IMP 20th Anniversary Conference, Vienna, Austria. 05/08.
Babraham Institute. University of Cambridge, UK. 05/08
Institute of Molecular Medicine. University of Oxford, UK. 05/08
Max Delbruck Centre, Berlin. Germany. 06/08
XVII Wilsede Meeting, Wilsede Germany. 06/08
4th International Conference on Gene Regulation in Lymphocyte Development. Rhodes, Greece. 10/08.
38th Annual Meeting of the Japanese Society for Immunology. Kyoto Japan. 12/08.
Keystone Symposium on B cells. Taos NM, USA. 03/09

EXHIBIT 2

ELSEVIER

Mechanisms of Development 117 (2002) 305–309

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Gene expression pattern

The dynamic expression pattern of B lymphocyte induced maturation protein-1 (Blimp-1) during mouse embryonic development

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Abstract

We have analyzed the spatial and temporal patterns of B lymphocyte-induced maturation protein-1 (Blimp-1) expression during mouse embryonic development. Blimp-1 expression is induced early in the anterior definitive endoderm, mesoderm of head process, and prechordal plate. In ectoderm-derived tissues at later stages, Blimp-1 expression is found in the primitive photoreceptors of neural retina, in differentiated epithelial cells of epidermis, tongue, oral and nasal cavities, and in the precursors of internal root sheaths of hair follicles. In mesoderm-derived tissues, Blimp-1 expression is observed in splanchnopleure, a subset of somatopleure-derived cells in limb buds, and myotomes of somites. Blimp-1 is also expressed in mesenchyme of developing hand plates, digits, branchial arches, nasal processes, and external genitalia. Blimp-1 is present in mesenchyme-derived chondroblasts, supporting cells of taste buds, and papilla of teeth, hair follicles and taste buds. In endoderm-derived tissues, Blimp-1 expression in the foregut region is restricted to a subset of epithelial cells at the headfold stage while expression in the endodermal epithelium of midgut and hindgut persists from the headfold stage to birth. Finally, Blimp-1 is expressed in the migrating primordial germ cells.

Keywords: Photoreceptors; Head process; Anterior definitive endoderm; Primordial germ cells; Splanchnopleure; Somatopleure; Myotome; Chondroblast; Limb; Digit; Genitalia; Nasal process; Branchial arches; Dental/lingual/dermal papilla; Taste bud; Internal root sheath; Intestine

1. Introduction

The Krüppel-type zinc-finger protein B lymphocyte-induced maturation protein-1 (Blimp-1) drives terminal differentiation of B lymphocytes (Turner et al., 1994), triggers macrophage differentiation (Chang et al., 2000), and is expressed in differentiated epithelial cells of adult mice (Chang and Calame, unpublished data). In B cells and macrophages, Blimp-1 represses transcription of genes involved in proliferation, including c-*myc* (Lin et al., 1997) and other genes involved in B cell and macrophage identity (Shaffer et al., in press; Chang et al., unpublished). Blimp-1 is required for embryonic development in mice since targeted disruption of the Blimp-1 gene, *prdm1*, causes death at E8–E10 (Davis, M.M., personal communication). Blimp-1 homologues, identified in multicellular eukaryotes from human to *Caenorhabditis elegans*, are evolutionary conserved (Tunyaplin et al., 2000), and the homologues in *Xenopus* (Xblimp1) and sea urchin (SpKrox1) were shown to be required during early embryonic development (de Souza et al., 1999; Wang et al., 1996). Here, we employed monoclonal antiserum against Blimp-1 for immunohistochemical staining to study Blimp-1 expression during mouse embryonic development.

2. Results and discussion

2.1. Blimp-1 in early development

Blimp-1 is first detected at about E7 (Fig. 1A–C) in the head process region in the anterior definitive endoderm and mesoderm, and in the prechordal plate, consistent with previous observations by de Souza et al. (1999) using in situ hybridization. At about E8, Blimp-1 expression becomes restricted to the endodermal epithelial lining of the growing foregut pocket and its neighboring splanchnopleure (Fig. 1D, E). Furthermore, at E7–E8, Blimp-1 expression is also found in primordial germ cells (PGCs), which aggregate close to the base of allantois and posterior endodermal lining (Fig. 1A, F).

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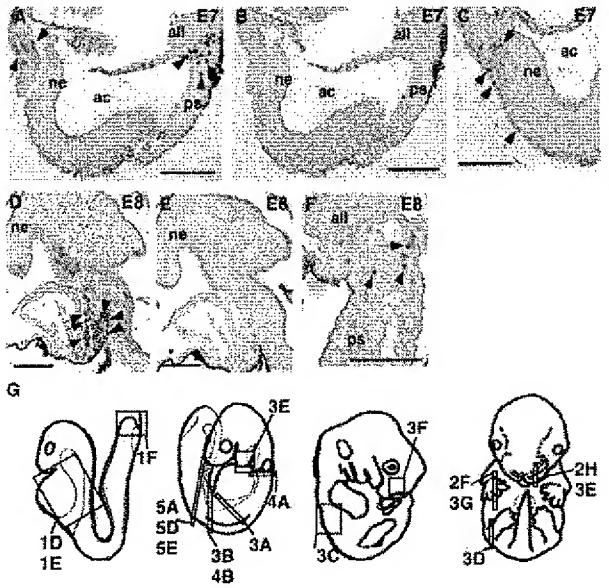

Fig. 1. Blimp-1 expression in early embryo. (A–C) Series of sagittal sections through E7 embryos with (C) section through the prechordal plate; anterior is to the left. (B) Control staining using mouse IgG1 standard. Blimp-1 expression is observed in the anterior definitive endoderm and mesoderm of head process and prechordal plate (C). Blimp-1 is also expressed in PGCs situated close to allantois (arrowheads). (D–F) Sagittal sections through an E8 embryo with (D) and (E) (control) at the anterior headfold region and (F) at allantois/primitive streak region; anterior is to the left. Blimp-1 is expressed in the splanchnopleure (D, arrows), endoderm of foregut pocket (D, arrowheads), and PGCs (F, arrows). (G) Embryo drawings from various developmental stages for orientation reference. Various lines indicate the plains of sectioning of the corresponding figures. The non-specific background staining close to the posterior end of (A–C), and along the outer lining of (D,E) correspond to extra-embryonic cell debris. Abbreviations: all, allantois; ac, amniotic cavity; ps, primitive streak; ne, neural epithelium. Scale bar indicates 1 μm.

2.2. Blimp-1 in ectoderm

In neural ectoderm-derived tissues, Blimp-1 expression is restricted to the primitive retinal neurons, which are concentrated in the outer neuroblastic layer from about E11 until birth (Fig. 2A–E). However, Blimp-1 is not expressed in brain, CNS, and other neural ectoderm-derived tissues. In surface ectoderm-derived tissues, Blimp-1 expression is restricted to the outer differentiated layer of stratified squamous epithelia covering the epidermis, oral cavity and tongue, and nasal cavity (Fig. 2F–H) but not in the basal cells of the epithelia. Blimp-1 is also expressed in the precursors of internal root sheaths of hair and vibrissae follicles (Fig. 3L, M) but not in the matrix cells enveloping dermal papilla.

2.3. Blimp-1 in mesoderm

Blimp-1 expression in mesoderm-derived tissue is detected in splanchnopleure from E8–E9 (Figs. 1D and 3A) and a subset of somatopleure-derived cells found within the limb buds at their initial elevation stage (Fig. 3A, B) but not at later stages (not shown). From E9–E11, Blimp-1 expression is restricted to myotomes of somites (Fig. 3B, C) but not in the differentiated myoblasts at later stages (not shown).

During organogenesis, Blimp-1 is expressed in chondroblasts found in perichondrium surrounding the cartilaginous models of developing bone (Fig. 3D). Blimp-1 is also expressed in mesenchymal cells at various sites of tissue formations. For example, Blimp-1 is expressed in mesenchymal cells along the edges of developing branchial arches and nasal processes (Fig. 3E, F), edges of developing hand plate and digits (Figs. 3G and 2G), and the tip of external genital tubercle (Figs. 3H and 4C). The expression appears when these protruding tissues begin to develop but diminishes when development is complete. During tongue development, Blimp-1 is expressed in lingual papillae and cells that support the taste buds (Figs. 3I and 2G). During tooth development, Blimp-1 is expressed in the dental papillae at the cap stage (Fig. 3J), and in primitive odontoblasts at

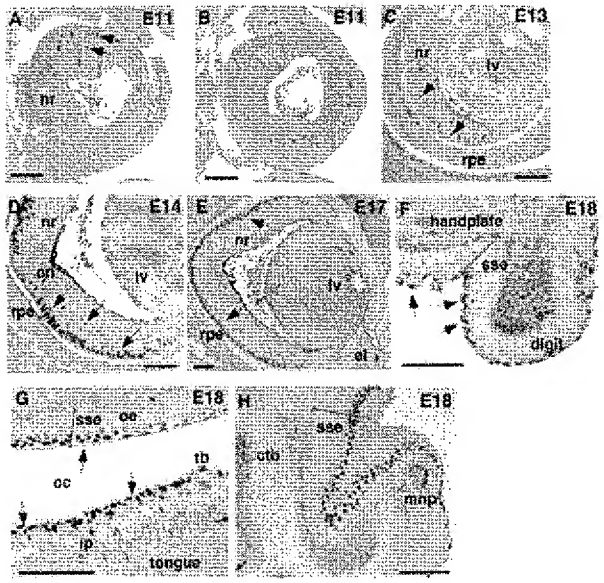

Fig. 2. Blimp-1 expression in ectodermal tissues. (A–E) Sagittal sections through the eyes of E11, E13, E14, and E17 embryos. (B) Negative control. Blimp-1 expression is observed in the outer neuroblastic layer of neural retina (arrows), and in the hair follicles in eyelid (white arrow in E). (F) Transverse section through the digits of an E18 embryo. Blimp-1-expressing cells are on the surface layer of epidermal epithelium (arrows), and also in the mesenchyme concentrating at the protruding digit (white arrow). (G) Sagittal section through the oral cavity of an E18 embryo. Blimp-1-expressing cells are located on the surface layer of stratified lingual epithelium (arrows) and also in the lingual papillae and supporting cells of taste buds (white arrows). (H) Sagittal section through the nasal process of an E18 embryo. Blimp-1 is expressed on the surface layer of the stratified squamous epithelium of nasal cavity (arrows), and also in the mesenchyme along the frontal edge of nasal processes (white arrow). Abbreviations: nr, neural retina; rpe, retinal pigment epithelium; lv, lens vesicle; on, optic nerve; el, eyelids; sse, stratified squamous epithelium; oc, oral cavity; oe, oral epithelium; lp, lingual papilla; tb, taste bud; ctb, cartilage of turbinate bones; mnp, median nasal process. Scale bar indicates 1 μm.

the bell stage (Fig. 3K); expression diminishes when dentin and enamel formation begins in newborn (not shown). During hair and vibrissae development, Blimp-1 is present in the dermal papillae throughout developmental stages (Fig. 3J, L, M) but diminishes when hair follicles become mature in the newborn (not shown).

2.4. Blimp-1 in endoderm

Blimp-1 expression in the foregut is restricted to a subset of endodermal epithelial cells at the headfold stage (Figs. 1D and 4A) but is not observed in its derivatives, like lungs, liver, or thymus (not shown). Blimp-1 expression in the foregut-derived tissues reappears later at about E18 in esophagus in the differentiated layer of stratified squamouse esophagus epithelium (Fig. 4E). Blimp-1 expression in the endodermal epithelial lining of midgut and hindgut, however, persists from E9 through till birth (Fig. 4B–D).

2.5. Blimp-1 in primordial germ cells

Blimp-1-expressing PGCs are observed among the endodermal cells of yolk sac near the allantois at about E7–E8 (Fig. 1A, D). Subsequently from E9–E11, these large, spherical Blimp-1-expressing PGCs migrate along the dorsal mesentery of the hindgut to the urogonadal ridges, and aggregate in the primitive gonads at about E11–E12 (Fig. 5A–F). The identity of PGCs has been confirmed by the expression of SSEA-1 (Stage-specific mouse embryonic antigen), a marker for PGCs from E9.5–E14.5 (Donovan et al., 1986). However, Blimp-1 expression in PGCs is rapidly terminated after E13 (not shown).

3. Materials and methods

3.1. Antibody specificity

The monoclonal antibody, 3H2E8, was obtained after immunization with a peptide (amino acids 199–409) from mouse Blimp-1. The specificity of 3H2E8 was confirmed by positive immunocytostaining of endogenous Blimp-1 and ectopically expressed Blimp-1 in cells (not shown) and negative staining following pre-absorption of 3H2E8 with Blimp-1 peptide (amino acids 199–409) (not shown).

3.2. Immunohistochemistry

3.2.1. Fixation and impregnation

BALB/c and C57BL6 mice embryos from various stages of development were dissected from euthanized pregnant

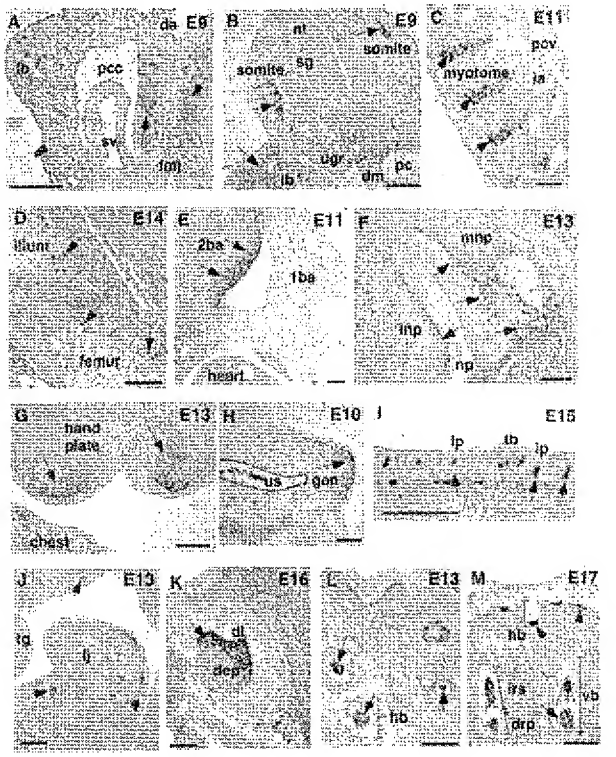

Fig. 3. Blimp-1 expression in mesodermal tissues. (A) Transverse section through the middle abdomen of an E9 embryo. Blimp-1 is expressed in the splanchnopleure (arrows) adjacent to the foregut–midgut junction, and in a few somatic mesodermal cells within the limb bud (white arrow). (B) Transverse section through the lower abdomen of an E9 embryo. Blimp-1 is expressed in the myotomes of somites (arrows) and in the mesodermal cells within the limb bud. Blimp-1 expression is also observed in PGCs aggregated in the urogenital ridge (white arrows). (C) Para-sagittal section through the lower back of an E11 embryo. Blimp-1 is expressed in the myotomes of somites (arrows). (D) Para-sagittal section through the cartilaginous models of developing iliac bone and femur of an E14 embryo. A few scattered Blimp-1-expressing cells are observed in the perichondrium around the cartilaginous bone models (arrows). (E) Para-sagittal section through the upper trunk of an E11 embryo along the mandibular component of first and second branchial arches. Blimp-1 is expressed in mesenchyme along the edge of the branchial arches. (F) Sagittal section through the developing nasal processes and nasal pit of an E13 embryo. Blimp-1 is expressed in the mesenchyme along the edge of developing nasal processes (arrows). (G) Section through the hand plate of an E13 embryo. (H) Sagittal section through the external genital tubercle of an E10 embryo. Blimp-1 is expressed in the mesenchymal cells concentrating at the tip of the protruding genital eminence (arrows). (I) Sagittal section through the tongue of an E15 embryo. Blimp-1 expression is observed in the mesenchyme of lingual papillae (arrows) and the supporting cells of taste buds (white arrows). (J,K) Sagittal sections through the lower jaws of E13 and E16 embryos. Blimp-1 expression is observed in the mesenchyme of dental papillae bordering the dental lamina (arrows). In (J), Blimp-1 expression is also seen in the mesenchyme of developing dermal papillae of hair follicles at the external part of lower jaw (white arrows). (L,M) Para-sagittal sections through the frontal nasal prominence region of E13 and E17 embryos. Blimp-1 is expressed in the dermal papillae of vibrissae (arrow) and hair follicles (arrowheads) and also in the precursors of internal root sheath (white arrow). Abbreviations: pcc, pericardial cavity; fmj, foregut–midgut junction; da, dorsal aorta; lb, limb bud; sv, sinus venosus; nt, neural tube; sg, spinal ganglion; ugr, urogenital ridge; dm, dorsal mesentery; pc, peritoneal cavity; pcv, posterior cardinal vein; ia, intercostals arteries; 1ba, first branchial arch; 2ba, second branchial arch; mnp, median nasal process; lnp, lateral nasal process; np, nasal pit; gen, genital tubercle; us, lumen of urogenital sinus; tb, taste bud; lp, lingual papilla; tg, tongue; lj, lower jaw; dl, dental lamina; dep, dental papilla; hb, hair bulb; vb, vibrissae bulb; drp, dermal papilla; irs, internal root sheath. Scale bar indicates 1 μm.

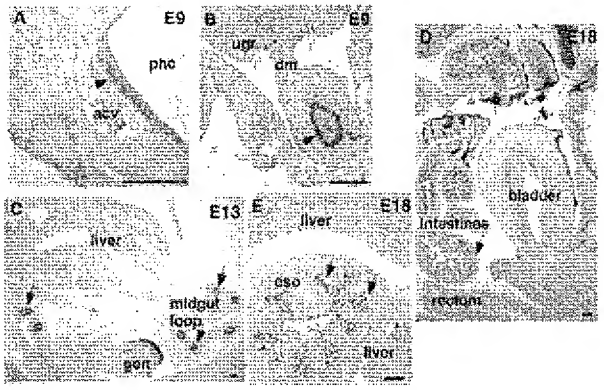

Fig. 4. Blimp-1 expression in endodermal tissues. (A) Transverse section through the pharynx cavity of an E9 embryo. Blimp-1 is expressed in the endodermal epithelium of foregut as indicated (arrow). (B) Transverse section through the lower abdomen of an E9 embryo. Blimp-1 is expressed in the endodermal epithelium of midgut (arrow), and PGCs in the urogenital ridge and dorsal mesentery (white arrows). (C) A global view of a section through the frontal abdomen of an E13 embryo. Blimp-1 expression is observed in the endodermal epithelial of midgut loops, intestine (arrows). The expression is also found at the tip of genital tubercle (white arrow). (D) A global view of a section through the abdomen of an E18 embryo. Blimp-1 is consistently expressed in the endodermal epithelial lining of intestine (arrows). (E) Section through the caudal part of esophagus. Blimp-1 expression is observed in the surface layer of stratified squamous endodermal epithelium. Abbreviations: phc, pharyngeal cavity; acv, anterior cardinal vein; dm, dorsal mesentery; ugr, urogenital ridge; gen, genital tubercle; eso, esophagus. Scale bar indicates 1 μm.

females, washed in ice-cold phosphate-buffered saline (PBS) before transferring into freshly prepared ice-cold 4% paraformaldehye (Sigma, prepared in PBS, pH 7.4) and fixed overnight at 4°C on a rocker. Paraformaldehye was sequentially replaced with ice-cold 1 × PBS, 0.85 % NaCl (saline), 1:1 ratio of ethanol:saline, and twice in 70% ethanol for 30 min each at 4°C on a rocker. The subsequent dehydration, embedding, and sectioning steps were performed by the Histology Facility of Columbia University.

3.2.2. Preparation for staining

Slides with 3–5-μm sections were deparaffinized twice in xylene for 5 min each, washed thrice in 100% ethanol for 5 min each, and subsequently rehydrated in 95, 75, 50, 30% ethanol, and distilled water for 5 min each. Slides were transferred into a plastic slide holder (VWR), immersed in a 1000-ml beaker with 800 ml of 10 mM EDTA, pH 7.8. The beaker was covered with Saran wrap punched with holes, boiled vigorously for 8 min at 100% power, and simmered for 15 min at 30% power. The slides were allowed to cool to room temperature for 30–60 min, transferred to staining dishes and washed twice in TBS-T (0.05 M Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20). Slides were then blotted for 10–30 min in egg white (one egg white in 100 ml PBS, 0.1% NaN_3), washed in TBS-T, blotted again in 5% milk (in TBS-T) for 10–30 min, and washed twice and kept in TBS-T.

3.2.3. Immunostaining

Slides were pre-incubated with 3% human serum in primary hybridization buffer (TBS, 2% BSA, and 0.1% NaN) for 5–10 min before applying primary antibody immediately after pouring off pre-hyb, and incubated in a TBS-humidified chamber for about 12–18 h at 4°C. Blimp-1 monoclonal antibody, 3H2E8, was diluted at 1:50 to 1:100 in primary hybridization buffer. Isotype-matched mouse IgG1 antibody standard unlabeled (Southern Biotech.) was used as a negative control. Slides were then washed six times in TBS-T, 5 min each, before incubating with the secondary antibody, anti-mouse IgG1 conjugated with alkaline phosphatase (Southern Biotech.) at 1:1000 dilution in the secondary hybridization buffer (TBS, 5% human serum, 10% egg white, 0.5% milk) for 2 h at room temperature. After incubation, slides were again washed six times in TBS-T for 5 min each. Color reaction was performed by incubating slides in NBT/BCIP solution (Roche, NBT and BCIP solutions or NBT/BCIP ready-to-use tablets following manufactures' protocol) for 1–18 h at room temperature in the dark. The color reaction was stopped by washing/rinsing slides in tap water several times before mounting with glycerol gelatin (Sigma).

For double staining with a second primary antibody, slides (after washing in tap water) were put back in beaker with 800 ml of 10 mM EDTA at pH 7.8, boiled for 8 min at

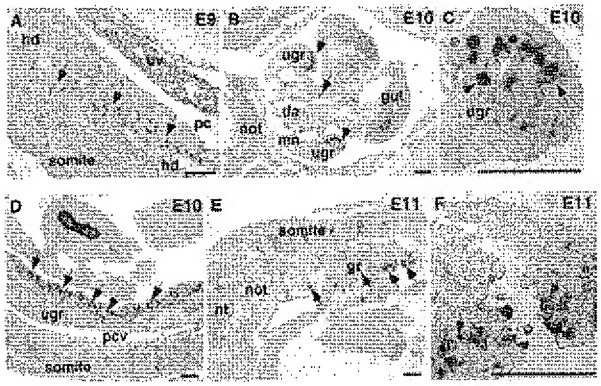

Fig. 5. Blimp-1 expression in PGCs. (A) Mid-sagittal section through the abdomen, across the hindgut diverticulum region. Blimp-1-expressing PGCs are scattered in the dorsal mesentery along the hindgut diverticulum (arrows). (B) Transverse section through the lower abdomen of an E10 embryo. (C) An enlarged view of a part of urogenital ridge of (B). PGCs expressing Blimp-1 (blue-purple nuclear staining) and SSEA-1 (red surface staining) are aggregated in the urogenital ridge while a few still scatter in the dorsal mesentery (arrows). On the other hand, Blimp-1 expressing endodermal epithelium of midgut does not express SSEA-1 (white arrow). (D,E) Para-sagittal sections through the lower abdomen of E10 and E11 embryos. (F) An enlarged view of the primitive gonadal ridge of (E). Again, PGCs expressing Blimp-1 (blue-purple nuclear staining) and SSEA-1 (red surface staining) are either scatter in the dorsal mesentery or aggregate in the primitive gonadal ridge. Blimp-1-expressing myotomes and epithelium of hindgut are negative for SSEA-1 staining (white arrows). Abbreviations: hd, hindgut diverticulum; pc, peritoneal cavity; uv, umbilical vein; gr, genital ridge; mn, mesonephros; da, dorsal aorta; dm, dorsal mesentery; ugr, urogenital ridge; pcv, posterior cardinal vein; not, notochord; nt, neural tube; gr, gonadal ridge. Scale bar indicates 1 μm.

100% power and simmered for 15 min at 30% power to inhibit alkaline phosphatase activity from anti-mouse IgG1-AP. After cooling down to room temperature for 30–60 min, slides were washed twice in TBS-T, pre-incubated with 3% human serum in TBS-humidified chamber before applying the second primary antibody and was incubated overnight at 4°C. The primary antibody, MC-480 supernatant (containing antibody against SSEA-1, purchased from DSHB, University of Iowa), was used at 1:5 dilution. The NS-1 supernatant (DSHB) was used as negative control. The subsequent washing, secondary antibody incubation, color reaction, and mounting were essentially the same as previous. The secondary antibody used was anti-mouse IgM-AP, and the AP substrate used for color reaction was Sigma Fast™ Fast Red TR/Naphthol AS-MX Tablet Sets (Sigma) following manufacturer's protocol.

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EXHIBIT 3

Plasma cell differentiation requires the transcription factor XBP-1

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Considerable progress has been made in identifying the transcription factors involved in the early specification of the B-lymphocyte lineage. However, little is known about factors that control the transition of mature activated B cells to antibody-secreting plasma cells. Here we report that the transcription factor XBP-1 is required for the generation of plasma cells. XBP-1 transcripts were rapidly upregulated *in vitro* by stimuli that induce plasma-cell differentiation, and were found at high levels in plasma cells from rheumatoid synovium. When introduced into B-lineage cells, XBP-1 initiated plasma-cell differentiation. Mouse lymphoid chimaeras deficient in XBP-1 possessed normal numbers of activated B lymphocytes that proliferated, secreted cytokines and formed normal germinal centres. However, they secreted very little immunoglobulin of any isotype and failed to control infection with the B-cell-dependent polyoma virus, because plasma cells were markedly absent. XBP-1 is the only transcription factor known to be selectively and specifically required for the terminal differentiation of B lymphocytes to plasma cells.

Production of antibodies by B lymphocytes is critical for the successful removal of pathogens. Once B lymphocytes encounter antigens, a complex series of activation and maturation events drives the generation of memory B cells and antibody-secreting plasma cells[1–3]. The initial activation of B cells is intimately linked to the ligation of cell-surface receptors and stimulation by cytokines. CD40 is a vital cell-surface molecule whose engagement on B cells results in proliferation, cell survival and the preferential formation of memory cells rather than plasma cells[4]. In addition, cytokines play an essential role in providing signals that influence cell proliferation (for example, interleukin-2 (IL-2), IL-5 and IL-6), protection from apoptosis (IL-4), choice of immunoglobulin isotype during class switching (for example, IL-4 enhances production of IgE), and choice of memory- or plasma-cell phenotype (IL-6 and IL-10)[3,5–13]. From such stimuli, IgM-secreting plasma cells are generated by T-cell-independent B-cell responses as well as B-cell activation in the extrafollicular zones of lymph nodes, whereas memory cells and non-IgM-secreting plasma cells result from immunoglobulin class switching and somatic hypermutation in germinal centres of lymph nodes[10].

Transcription factors ultimately integrate the signals from cytokine receptors and other activating cell-surface molecules to play essential roles in B-cell maturation and activation[14]. When a B cell encounters antigen, the initial signalling events depend on the actions of transcription factors such as BSAP (B-cell-specific activator protein), NF-κB/Rel, Aiolos and the Ets family members PU.1 and Spi-B[14,15]. In contrast to these early steps in B-cell development and activation, little is known about the signals that control the transition from an activated B cell to a plasma cell. Plasma cells specialize in the synthesis and secretion of immunoglobulin, and they downregulate a large number of cell-surface molecules (B cell receptor, major histocompatibility complex (MHC) class II, CD20, CD44, B220) and transcription factors (CIITA, BSAP/Pax-5)[16,17] that are not vital for the terminally differentiated phenotype. Among the few markers that distinguish plasma cells from mature B cells are cell-surface Syndecan-1, as well as expression of transcription factors IRF-4 (interferon regulatory factor-4) and Blimp-1 (B-lymphocyte-induced maturation protein-1). To date, the only transcription factor known to drive B-cell differentiation to plasma cells is Blimp-1 (also called PRDF1-BF1)[18–20]. This zinc-finger protein is specifically expressed in mature B cells and plasma cells but not in memory cells. Overexpression of Blimp-1 in BCL1-3B3 lymphoma cells causes the appearance of an early plasma-cell phenotype, including J-chain expression and IgM secretion[18–20]. One aspect of the mechanism by which Blimp-1 promotes generation of plasma cells is the repression of c-Myc, thereby allowing the B cell to exit the cell cycle and undergo terminal differentiation[21–23].

XBP-1 (X-box-binding protein-1) is a basic-region leucine zipper protein in the CREB/ATF (cyclic AMP response element binding protein/activating transcription factor) family of transcription factors, found ubiquitously in adult tissues but preferentially expressed in fetal exocrine glands, osteoblasts, chondroblasts and liver[24–26]. XBP-1 is essential for the growth of hepatocytes, as XBP-1-deficient embryos die *in utero* from severe liver hypoplasia and a resulting fatal anaemia[27]. In human multiple myeloma cells, XBP-1 was selectively induced by treatment with IL-6 and was implicated in the proliferation of malignant plasma cells[28]. Transient expression studies have shown that the XBP-1 promoter is strongly downregulated by the transcription factor BSAP/Pax-5, which may account for the high level of XBP-1 expression seen in some plasma cell lines, a developmental stage where BSAP is no longer present[29].

As XBP-1-deficient mouse embryos die *in utero*, it was not possible to study immune functions in these animals. Therefore, the RAG-2 (recombination-activating gene-2) complementation system was used to analyse XBP-1-deficient lymphocytes from adult chimaeric animals[30]. These lymphoid chimaeras displayed a severe defect in the generation of plasma cells and therefore of immunoglobulin secretion. This identifies XBP-1 as a transcription factor essential for the terminal differentiation of B lymphocytes.

High XBP-1 in plasma cells at inflamed sites

We have previously described the regulated expression of XBP-1 transcripts in B cells at various stages of development, and noted the

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extremely high level of XBP-1 transcripts in several myeloma cell lines[29]. The disease rheumatoid arthritis offered a source of clinical material to study XBP-1 expression *in vivo* in primary cells in a condition characterized by the proliferation of inflammatory tissue and the infiltration of inflammatory cells, including, in many cases, large numbers of plasma cells[31]. With an *in situ* antisense probe for XBP-1, we demonstrated strong hybridization to plasma cells in rheumatoid synovium from two different patients (Fig. 1a, d), whereas sense probes showed no specific hybridization (Fig. 1b, e). High-power microscopy confirmed the presence of numerous plasma cells in areas of XBP-1 expression (Fig. 1c, f). These data demonstrate high-level expression of XBP-1 transcripts in the plasma cell infiltrates of an inflammatory disease.

Upstream and downstream roles of XBP-1

Activated B cells are driven to become plasma cells by signalling through the CD40 receptor or through mitogens such as lipopolysaccharide (LPS). These two stimuli also drive the upregulation of transcripts encoding XBP-1 in purified B cells (Fig. 2a). The BCL1-3B3 cell line is a highly activated B-cell line that can be driven to an early plasma-cell stage by treatment with IL-2 and IL-5 (refs 32, 33). We tested whether XBP-1 could also drive B-cell differentiation in this model. BCL1-3B3 cells express XBP-1 transcripts at baseline levels, and this does not increase after treatment with IL-2 and IL-5. By analysis with fluorescence-activated cell sorting (FACS), cells overexpressing a bicistronic XBP-1–GFP (green fluorescent protein) construct but not the GFP vector alone showed signs of further maturation: a decrease in CD44 levels and the emergence of Syndecan-1-positive cells (Fig. 2b). An identical effect has been described for the ectopic expression of transcription factor Blimp-1 in BCL1-3B3 cells[19].

XBP-1/RAG-2-deficient chimaeric mice

XBP-$1^{-/-}$ embryonic stem cells of mice were identified on Southern blots of genomic DNA by the absence of a wild-type XBP-1 band and the presence of two disrupted alleles (Fig. 3a). After injection into RAG-2-deficient blastocysts, XBP-1-deficient cells contributed to reconstitution of peripheral blood B and T lymphocytes in approximately half of the mice born. In these reconstituted animals, XBP-$1^{-/-}$ embryonic stem cells contributed heavily to lymphoid organs, variably to heart, lung, kidney, muscle and bone marrow but minimally to liver (Fig. 3b), consistent with the essential role of XBP-1 in hepatocyte development seen in XBP-$1^{-/-}$ embryos. The reconstitution of lymph nodes and spleen in XBP-1/RAG-$2^{-/-}$ animals resulted in normal-sized organs indistinguishable from those of control 129/SvImJ mice. Chimaeric mice had normal numbers (Fig. 3c), percentages and phenotype of B and T (not shown) lymphocytes on FACS analyses as assessed by expression of B220, IgM and IgD (Fig. 3d). Peritoneal $CD5^+$ B-1 lymphocytes were also found in equal numbers in chimaeric and control animals (not shown).

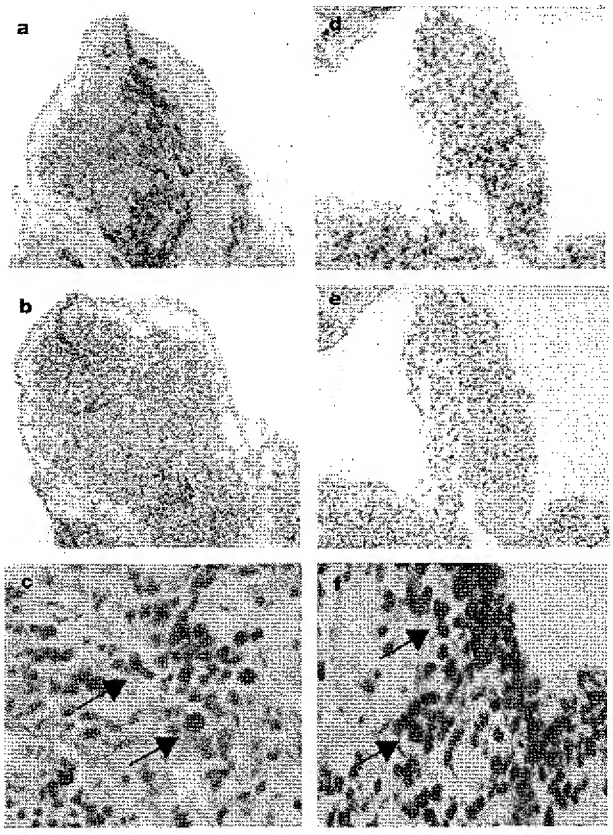

Figure 1 XBP-1 expression in rheumatoid synovium. **a**, **d**, *In situ* hybridization with an antisense XBP-1 probe on synovium from two patients with rheumatoid arthritis. **b**, **e**, Sections from the tissue blocks were hybridized with a sense XBP-1 probe (**e**) and with an unrelated (**b**) sense control probe as controls. **c**, **f**, High-power views of sections stained with haematoxylin and eosin show areas of numerous plasma cells (arrows) in regions where XBP-1 signals were most intense.

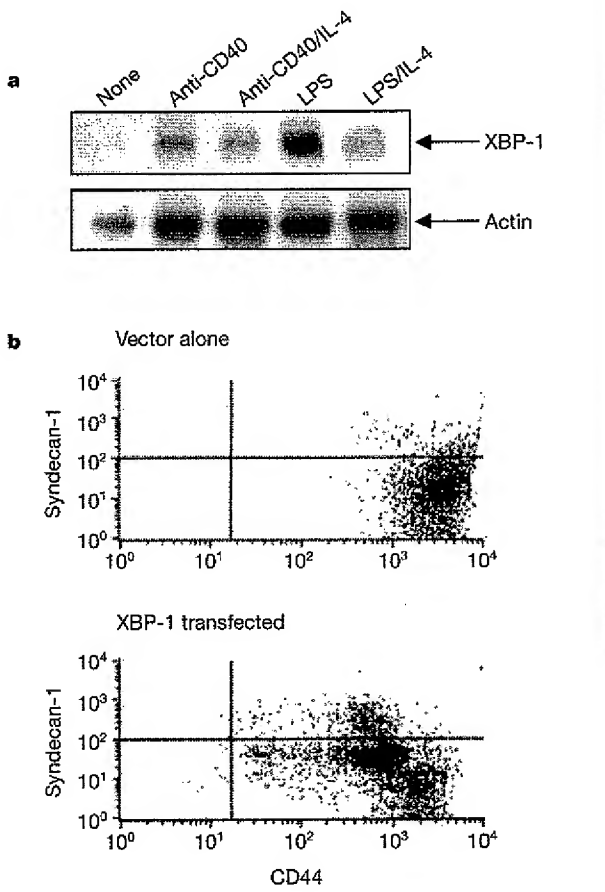

Figure 2 Induction of XBP-1 is upstream and downstream of signals that drive plasma-cell differentiation. **a**, Induction of XBP-1 transcripts in wild-type B lymphocytes. A northern blot of RNA derived from cells stimulated *in vitro* for 4 d is shown. **b**, XBP-1 drives differentiation of an activated, mature B-cell line. BCL1-3B3 cells were transfected with expression constructs containing no insert (vector alone) or XBP-1 cDNA. Cells were sorted for GFP expression and after 2 d of growth, a FACS analysis was performed. Differentiating cells are recognized by upregulation of cell-surface Syndecan-1 and downregulation of CD44.

Low immunoglobulin from XBP-1$^{-/-}$ B cells

To assess the major function of B lymphocytes, immunoglobulin levels were assayed by enzyme-linked immunosorbent assay (ELISA) in the sera of multiple XBP-1/RAG-2$^{-/-}$ mice and control 129/SvImJ mice. Baseline serum immunoglobulin levels were markedly lower in XBP-1/RAG-2$^{-/-}$ animals for all immunoglobulin subtypes tested, including IgM, with a small amount of IgG2a detected in some of the mice (Fig. 4a). To test the intrinsic potential of XBP-1-deficient B cells for immunoglobulin production after stimulation *in vitro*, splenocytes or purified B cells were treated *in vitro* for 4 days with a stimulus that directly drives immunoglobulin secretion from B cells in the absence of other cell types: the B-cell mitogen LPS. When immunoglobulin levels were determined in culture supernatants, all tested immunoglobulin subtypes were again found at substantially lower levels in XBP-1$^{-/-}$ samples than in control samples (Fig. 4b). To directly demonstrate the effect of XBP-1 in driving immunoglobulin secretion in B cells, retroviral transduction was used to re-express XBP-1 in XBP-1-deficient B cells. Compared with the introduction of the retroviral vector alone, the addition of XBP-1 more than doubled the secretion of IgM (Fig. 4c). Therefore, the XBP-1 transcription factor is very important in the elaboration of immunoglobulins by B lymphocytes.

In vitro phenotype of XBP-1$^{-/-}$ B cells

Given the functional defect in XBP-1$^{-/-}$ terminal B-cell differentiation, we investigated the phenotype of B-cell activation *in vitro*. B cells were stimulated *in vitro* with anti-CD40 antibody, or anti-CD40 and IL-4, for up to 4 days. The B-cell populations were activated similarly, as judged by analysis of the cell-surface markers MHC class II (I–A), B7.2, CD25, CD69 and CD95 (Fig. 5), and CD19, CD21, CD40, CD44, CD62L and CD80 (not shown). Cell proliferation was the same in XBP-1-deficient and control samples, as judged by equivalent cell counts on days 1–4 of *in vitro* culture (Fig. 6a). The activation of T cells in response to anti-CD3 was also normal as judged by proliferation and expression of CD62L and CD40L (not shown). Therefore, no significant differences were found in the activation of XBP-1-deficient B or T lymphocytes. *In vitro* stimulation with LPS was used to induce class-switch recombination in B cells, a process that is regulated separately for membrane and secreted forms. Class-switch recombination was equivalent in XBP-1$^{-/-}$ and control samples, as judged by FACS analysis of cell-surface immunoglobulin and by polymerase chain reaction with reverse transcription (RT-PCR) with primers that selectively recognize germline IgG2b and IgG3 and all IgM membrane and secreted species (Fig. 6b), thus making a direct role for XBP-1 in regulating immunoglobulin transcription unlikely. However, XBP-1$^{-/-}$ B cells were less differentiated as judged by decreased expression of J-chain, which is required for assembly of IgM and IgA in plasma cells, as well as increased expression of c-Myc, which becomes downregulated as B cells exit the cell cycle to differentiate terminally (Fig. 6c). The cytokine profile of XBP-1-deficient B cells cultured *in vitro* and stimulated *in vitro* with anti-CD40 antibody or LPS, with or without the addition of IL-4, showed no significant differences in the production of IL-6 or IL-10 when compared with control cells (not shown). Therefore, XBP-1-deficient B cells are present in normal numbers and can be activated *in vitro* to undergo proliferation, cell-surface activation-marker expression, class switch recombination, and cytokine secretion at normal levels.

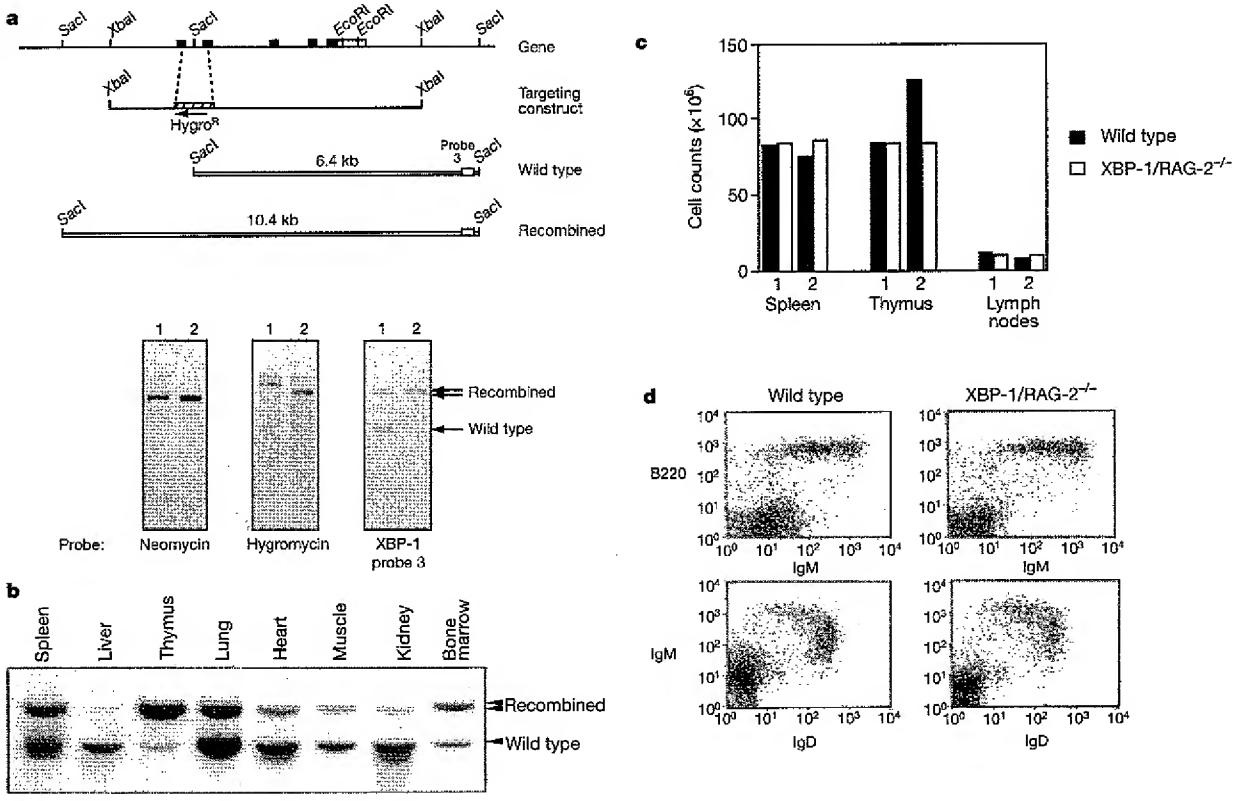

Figure 3 Gene targeting to generate XBP-1-deficient cells. **a**, The XBP-1 gene was disrupted by homologous recombination using a vector containing a hygromycin drug-resistance cassette in place of parts of XBP-1 exons 1 and 2 as well as the intervening intronic DNA. This vector was electroporated into embryonic stem cells already containing a disruption of one XBP-1 allele. **b**, Southern blotting of tissues from mice deficient in XBP and RAG-2. Cells with a disrupted XBP-1 gene make up much of the lymphoid organs, whereas their contribution to other organs varies widely. **c**, Cell counts of lymphoid organs of XBP-1/RAG$^{-/-}$ and wild-type mice. **d**, Phenotype of XBP-1-deficient B lymphocytes. FACS analysis with B220, IgM-FITC and IgD-PE of control 129/SvImJ (wild type) and XBP-1/RAG-2$^{-/-}$ splenocytes.

We conclude that other mechanisms must account for the impaired immunoglobulin production by XBP-1$^{-/-}$ B cells.

Few plasma cells in the absence of XBP-1

The absence of germinal-centre formation can lead to impaired production of immunoglobulin, as evidenced by many mouse mutant models[34–37]. To assess whether XBP-1-deficient B cells respond to activating stimuli *in vivo*, mice were immunized with 2,4-dinitrophenyl (DNP)-albumin and their draining lymph nodes were gathered after 9 days. Histologic analysis demonstrated normal germinal-centre formation in XBP-1/RAG-2$^{-/-}$ animals (Fig. 7a). This indicates that many of the B-cell functions assayed *in vitro*, such as cell proliferation and activation, were also intact *in vivo*.

Histological analysis of organs from XBP-1/RAG-2$^{-/-}$ animals, however, revealed a striking absence of plasma cells. Sections of jejunum from XBP-1/RAG-2$^{-/-}$ and control mice showed approximately 70-fold fewer plasma cells in the former (Fig. 6b). Twenty high-power fields (×50 objective) of lymphoid tissue in spleen were examined from three immunized wild-type mice and three immunized XBP-1$^{-/-}$ chimaeric mice. The average number of plasma cells in the wild-type mice per field was 5, whereas the average number in the knockout mice was 0.2. In addition, it was noted that the rare plasma cells seen in XBP-1/RAG-2$^{-/-}$ animals generally lacked a perinuclear huff, indicating that these cells did not have a large Golgi apparatus and were probably not secreting large amounts of immunoglobulins. Histological examination of spleen and bone marrow revealed a similar striking absence of plasma cells in XBP-1/RAG-2$^{-/-}$ animals (not shown).

An additional indicator of plasma-cell differentiation is the presence of cells positive for Syndecan-1. Syndecan-1 is a cell-surface glycoprotein upregulated on terminally differentiated plasma cells but absent on mature B cells[38]. As predicted by the low level of immunoglobulin secretion, XBP-1$^{-/-}$ samples from mice immunized with DNP-albumin did not contain significant numbers of Syndecan-1-positive cells, whereas a significant increase above baseline was readily detected in the wild-type control (Fig. 7c). Therefore, XBP-1/RAG-2$^{-/-}$ animals display a severe defect in the generation of terminally differentiated plasma cells. The finding of very few plasma cells despite apparently normal germinal centres locates the primary site of XBP-1 action to the interval between full B-cell activation and terminal differentiation. It is not possible to more precisely localize the block as very few markers are available that distinguish activated mature B cells from early plasma cells.

XBP-1$^{-/-}$ B cells are unresponsive to antigen *in vivo*

Whereas the above experiments firmly establish an intrinsic defect in production of antibodies by B cells when activated *in vitro*, it is not clear to what extent this defect is relevant to B-cell function *in vivo*. The potential of XBP-1$^{-/-}$ B cells to secrete both T-cell-independent and -dependent antigen-specific immunoglobulin was therefore assessed by immunization of mice with TNP-Ficoll and TNP-CGG (chicken gamma-globulin), respectively. Antigen-specific immunoglobulin production by XBP-1$^{-/-}$ B cells in response to the T-cell-independent antigen TNP-Ficoll, which was primarily of the IgM and IgG3 isotypes, was measured on days 7, 14 and 21 after immunization and found to be virtually undetectable (Fig. 8a). This defect was equally apparent in mice immunized with the T-cell-dependent antigen TNP-CGG (Fig. 8b) at the same time

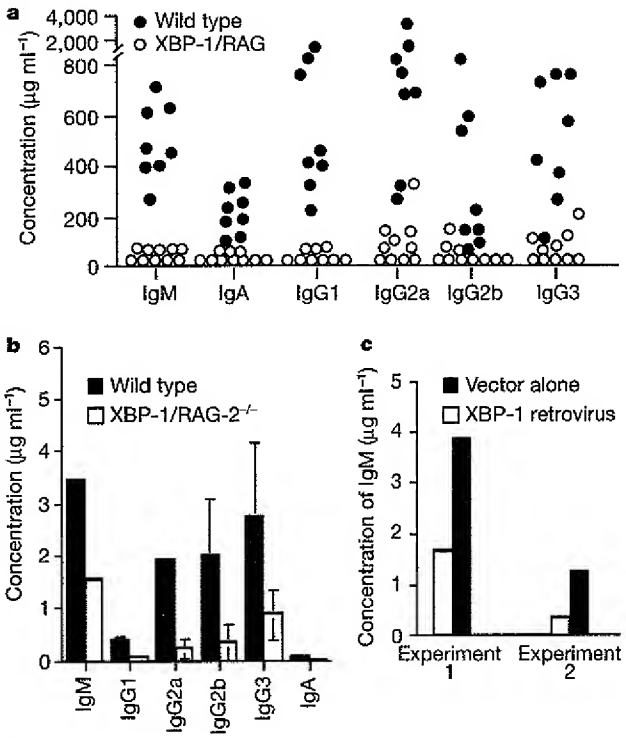

Figure 4 XBP-1 is required for immunoglobulin secretion. **a**, Baseline serum immunoglobulin levels in age-matched control 129/SvImJ mice or XBP-1/RAG-2$^{-/-}$ mice as measured by ELISA. **b**, B lymphocytes from 129/SvImJ or XBP-1/RAG-2$^{-/-}$ animals were cultured *in vitro* with LPS, and immunoglobulin levels in the supernatants were determined after 4 d of culture. **c**, Partial restoration of immunoglobulin secretion by XBP-1$^{-/-}$ B cells. After activation and transduction with a retroviral vector alone or with a retrovirus expressing XBP-1, cells were FACS sorted 24 h after transduction to include only GFP-positive cells that were actively transcribing the retroviral construct. XBP-1-deficient B cells were cultured *in vitro* for 3 days further and the supernatants were tested for IgM concentration.

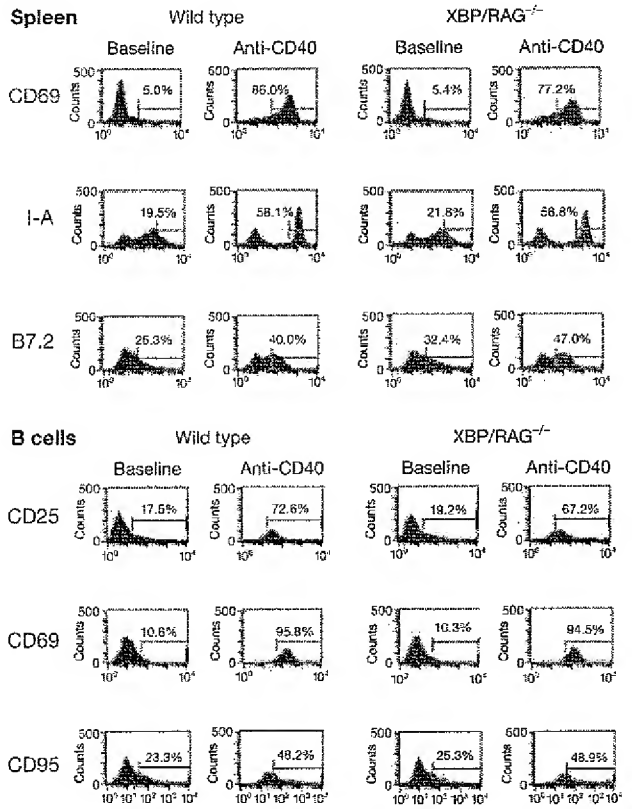

Figure 5 Surface activation markers (CD69, MHC class II (I-A), B7.2, CD25 and CD95) on B cells are normal in the absence of XBP-1.

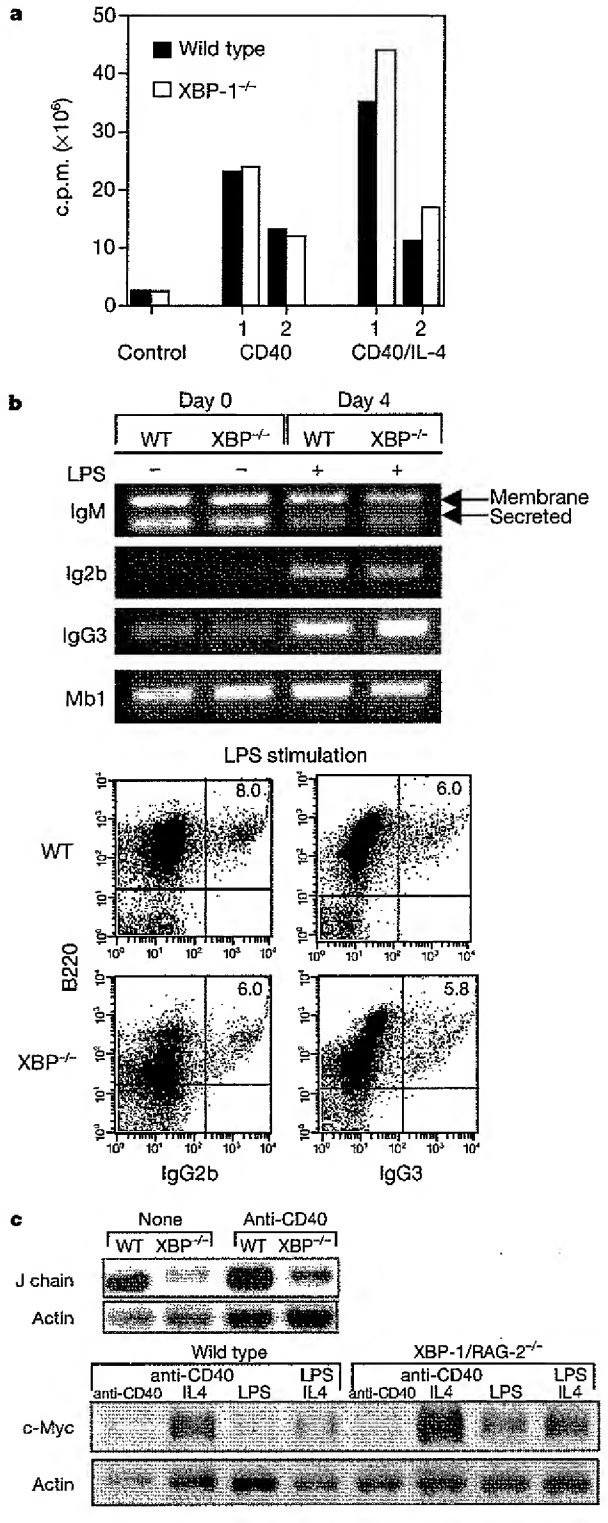

Figure 6 *In vitro* phenotype of XBP-1$^{-/-}$ B cells. **a**, B-cell proliferation is normal in XBP-1$^{-/-}$ cells. **b**, Class switch recombination is unaffected by the absence of XBP-1. FACS analysis of surface IgG, and RT-PCR analysis of all membrane and secreted IgM transcripts and IgG2b and IgG3 germline transcripts after LPS stimulation. WT, wild type. **c**, Diminished terminal differentiation of XBP-1-deficient B lymphocytes after *in vitro* stimulation for 4 d. Northern blots show that control cultures have undergone further differentiation than XBP-1-deficient cultures as XBP-1$^{-/-}$ B cells have decreased levels of J-chain transcripts and increased levels of c-Myc transcripts. B lymphocytes from 129/SvImJ or XBP-1/RAG-2$^{-/-}$ animals were cultured *in vitro* with anti-CD40 antibody, anti-CD40 plus IL-4, LPS or LPS plus IL-4.

points and involved all responding immunoglobulin subclasses including the IgM, IgG1 and IgG2a isotypes. These findings indicate that, although XBP-1-deficient B cells are generated in normal numbers, they fail to become antibody-secreting cells *in vivo* in response to antigen-specific, T-cell-independent or -dependent stimuli. The failure to respond to antigens that are not dependent on T-cell help clearly indicates an intrinsic B-cell defect in the secretion of immunoglobulin in the absence of XBP-1.

XBP-1$^{-/-}$ chimaeras are unresponsive to polyoma virus

We have determined that XBP-1$^{-/-}$ chimaeras do not mount an antibody response when immunized with either T-cell-independent or -dependent antigens, and established by *in vitro* assays that there is an intrinsic defect in the B-cell compartment. We next determined whether an immune response against pathogens that evoke protective humoral immune responses in addition to cell-mediated immune responses could be mounted in the absence of XBP-1. Viruses can be controlled exclusively by antibodies (rotavirus, polyoma virus, vesicular stomatitis virus), by antibodies and T cells (influenza, poliovirus, encephalomyocarditis), or exclusively by T cells (lymphocyte choriomeningitis virus, human immuno-

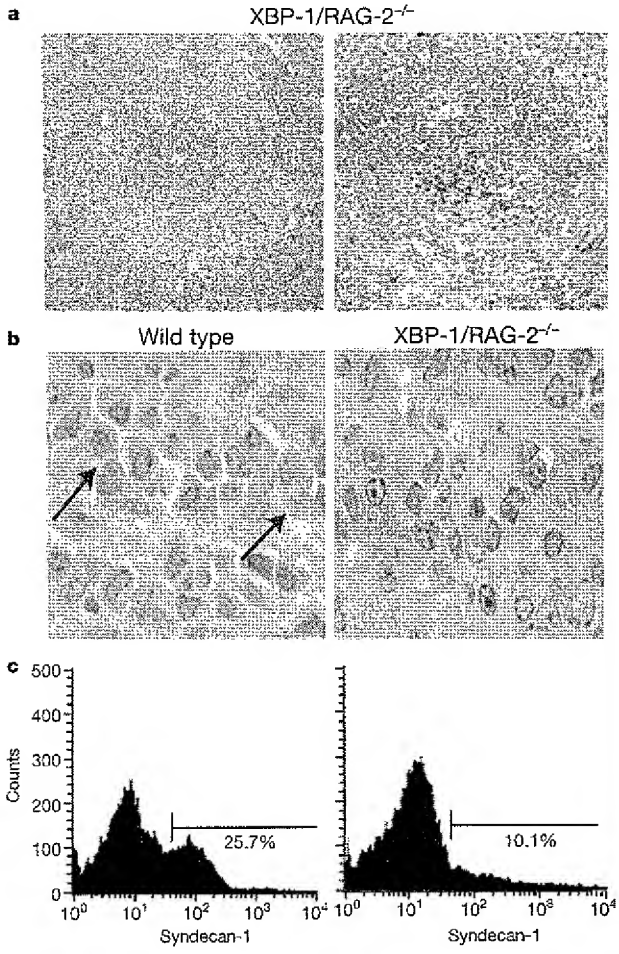

Figure 7 Decreased formation of plasma cells in the absence of XBP-1. **a**, Histological section of a germinal centre from a draining lymph node of an XBP-1/RAG-2$^{-/-}$ mouse immunized with DNP-albumin. The histologic appearance is normal. **b**, Sections of mouse jejunum showing a marked reduction in plasma cell numbers in XBP-1/RAG-2$^{-/-}$ animals. **c**, Lack of plasma-cell generation after immunization of XBP-1/RAG-2$^{-/-}$ mice. FACS analysis of B lymphocytes from control (left) and XBP-1/RAG-2$^{-/-}$ (right) animals showed an increase in Syndecan-1-positive cells (a marker for plasma cells) only in control samples.

deficiency virus, simian immunodeficiency virus)[39]. We therefore determined the IgM and IgG response of XBP-1$^{-/-}$ chimaeras to polyoma virus, a virus that is controlled by B cells, apparently in the absence of T-cell help[40]. B cells purified from wild-type mice or from XBP-1/RAG$^{-/-}$ chimaeras (or from irradiated recipients reconstituted with bone marrow or spleen from such chimaeras) were transferred into host severe combined immunodeficient (SCID) mice that were then infected with polyoma virus. As shown, XBP-1$^{-/-}$-transferred B cells did not confer protection to polyoma virus as evidenced by the failure of these reconstituted recipients to produce polyoma-virus-specific (VP1-specific) IgG (Fig. 8c) or IgM (not shown). These mice also had greatly impaired survival when compared with the control recipients.

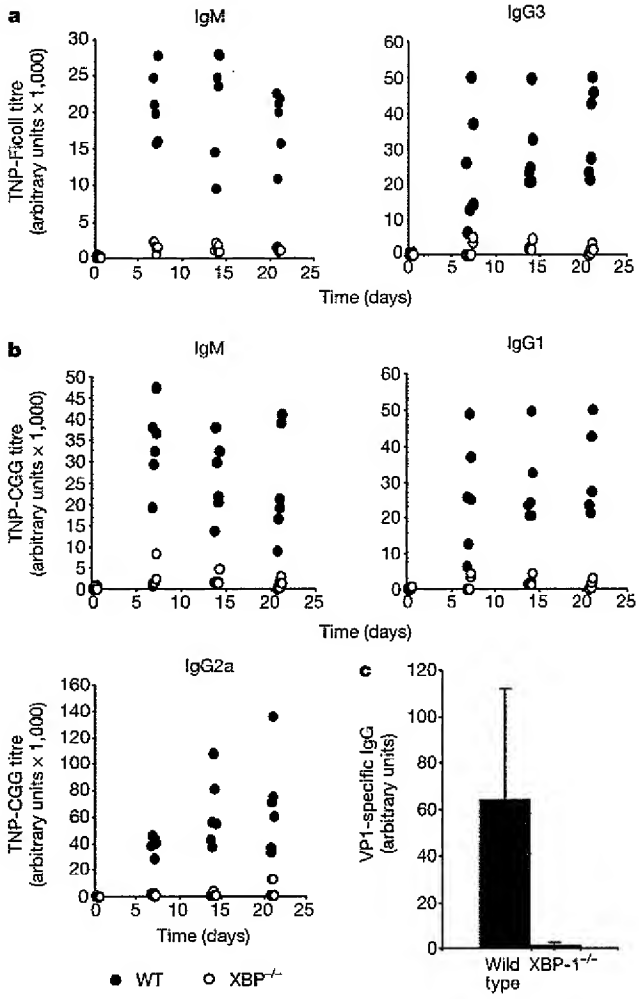

Figure 8 XBP-1$^{-/-}$ lymphocytes do not respond to T-cell-independent or -dependent antigens or to polyoma virus *in vivo*. **a**, T-cell-independent antibody production. Serum antibodies in response to immunizations with TNP-Ficoll were determined in wild-type 129/Sv and XBP-1/RAG-2$^{-/-}$ mice. Mice were immunized with 50 μg of TNP-Ficoll precipitated in alum. Serum was analysed for TNP-specific IgM and IgG3 antibodies on days 7, 14 and 21. Data points are arbitrary units of optical density for individuals ($n = 6$). **b**, T-cell-dependent antibody production. Serum antibodies in response to immunizations with TNP-CGG were determined in wild-type 129/Sv and XBP-1/RAG-2$^{-/-}$ mice. Mice were immunized with 100 μg of TNP-CGG precipitated in alum. Serum was analysed for TNP-specific IgM, IgG1 and IgG2a antibodies on days 7, 14 and 21. Data points are arbitrary units of optical density for individuals ($n = 6$). **c**, Polyoma-virus-specific IgG response of SCID mice reconstituted with XBP-1$^{-/-}$ B cells. VP1-specific IgG (day 21; tested in 1:50 to 1:400 dilution) of SCID mice reconstituted with T-cell-depleted splenocytes of control 129/Sv ($n = 3$) or XBP-1/RAG-2$^{-/-}$ ($n = 3$) mice. Values are expressed as arbitrary optical-density units at 450 nm, with background values (obtained from uninfected mouse serum) subtracted. The optical-density values obtained with uninfected mouse serum are below 0.08.

Discussion

XBP-1 is the only transcription factor known to be selectively and specifically required for the terminal differentiation of B lymphocytes to plasma cells. Its induction on B-cell activation and high-level expression in plasma cells coupled with the absence of plasma cells in XBP-1$^{-/-}$ lymphoid chimaeras identifies a single important site of action for XBP-1 in the terminal stages of the B lineage. Significantly, germinal centres were formed in the absence of XBP-1, placing the observed defect distal to the actions of several transcription factors shown to be essential for germinal-centre formation, including Bcl-3, Bcl-6, NF-κB/p52 and IRF-4 (refs 34–37). B cells that survive the selection, activation and differentiation steps that occur in germinal centres exit to become either memory B cells or plasma cells. XBP-1 is now known to be essential for this process, although other molecular details remain unknown. Recent histological studies have indicated that some cells destined to become plasma cells may already be identifiable in the germinal centre. The transcription factors Blimp-1 and IRF-4 are heavily expressed in plasma cells but have also been detected by specific antibodies in a small subset of light-zone germinal-centre B cells, probably representing cells exiting the germinal centre to differentiate to plasma cells[20,35]. Morphologically, such cells have had the phenotype of centrocytes or the more mature plasmablasts or plasma cells. Neither Blimp-1 nor IRF-4 is expressed in most germinal-centre B cells, indicating specific upregulation during terminal differentiation. However, IRF-4 is already known to be essential for the earlier steps of germinal-centre formation, and the role of Blimp-1 *in vivo* is still being elucidated.

We found that XBP-1$^{-/-}$ B cells could be activated but were blocked in terminal differentiation, a phenotype characterized by ongoing transcription of the proto-oncogene c-*myc*. XBP-1 and Blimp-1 have identical effects when overexpressed *in vitro*, leading to differentiation of Bcl-1 lymphoma cells but also to apoptosis of immature B-cell lines (refs 41 and 42; A.M.R. and L.H.G., unpublished observations). Such differences in responses, dependent on the stage of B-cell differentiation, have previously been noted following B-cell-receptor ligation. The underlying differences in signal transduction between immature and mature B cells remain poorly understood: B-cell-receptor ligation results in cell-cycle arrest and apoptosis of stimulated immature B cells[7,22,42], whereas mature B cells initiate expression of Egr-1, c-Fos and c-Myc and go on to activate and proliferate. Subsequent downregulation of the c-*myc* gene allows B cells to cease proliferating and to differentiate, instead. The repression of the c-*myc* promoter is an important mechanism of action for the complex of Blimp-1 and histone deacetylase in contributing to B-cell differentiation[21,23]. However, exiting the cell cycle is not sufficient to cause B-cell differentiation[43]. This indicates that as-yet-unknown XBP-1 or Blimp-1 target genes are essential in plasma-cell generation. However, the functions of XBP-1 and Blimp-1 are unique, as levels of Blimp-1 messenger RNA in activated B cells were not affected by the absence of XBP-1 (not shown) and thus Blimp-1 does not compensate for the absence of XBP-1. Further, XBP-1 does not directly repress activation of the c-*myc* promoter (A.M.R. and L.H.G., unpublished observations). Finally, whether Blimp-1 is involved in T-cell-dependent as well as T-cell-independent antibody production is controversial, whereas XBP-1 clearly is vital for both of these processes[21,44]. Therefore, XBP-1 acts downstream of Blimp-1 or through a separate pathway in its regulation of B-cell differentiation.

Our experiments therefore indicate that XBP-1 regulates a final common pathway of plasma-cell generation, regardless of the immune stimulus or the anatomic location of the immune response. Immunoglobulin production remained minimal in the absence of XBP-1, whether animals were immunized with T-cell-independent

antigen, T-cell-dependent antigen or polyoma virus. Furthermore, the populations of plasma cells that are normally found in lymph nodes, spleen, bone marrow or lamina propria of the gut were all severely reduced in the absence of XBP-1, indicating that the action of XBP-1 is fundamental to the mechanism of plasma-cell generation. We conclude that XBP-1 represents a molecular switch in the terminal differentiation of B cells. XBP-1 clearly is intrinsic in B-cell differentiation, yet low-level expression of XBP-1 is ubiquitous so there may well be additional roles for XBP-1 in the immune system, a possibility that we are currently exploring. □

Methods

Disruption of XBP-1

A genomic clone containing XBP-1 was disrupted by deletion of parts of exons 1 and 2 along with the intervening intron and by insertion of a cassette encoding a gene for hygromycin resistance. Embryonic stem cells from the 129 mouse strain with one allele of XBP-1 previously disrupted by the neomycin gene were transfected with the new construct and selected in hygromycin, neomycin and gancyclovir[37]. Surviving clones were screened by Southern blotting for the presence of two disrupted XBP-1 alleles and the loss of the wild-type allele. Appropriate embryonic-stem clones were injected into RAG-2-deficient blastocysts and implanted into pseudopregnant female mice as described[50]. The resulting mice were assayed for lymphoid reconstitution by FACS analysis of peripheral blood mononuclear cells using anti-CD3 and anti-B220 antibodies (PharMingen).

Antibodies and cell culture

FACS analysis was performed using antibodies to mouse CD69, MHC class II (I-A), CD3, B220, CD44 and Syndecan-1 (PharMingen). The BCL1-3B3 cell line (ATCC) was grown according to the supplied protocol. Transfection of the BCL1-3B3 cell lines was by electroporation at 290 V and 275 μF in a BioRad Electroporator. Cells were then sorted for GFP expression and after 2 d of growth, a FACS analysis was performed. Mouse splenocytes or purified B cells (B220+ magnetic bead selection, Miltenyi Biotech) were plated at 10^6 cells ml^{-1} and stimulated for up to 4 d with anti-CD40 (1 μg ml^{-1}), anti-CD40 plus IL-4 (10 ng ml^{-1}), LPS (20 μg ml^{-1}), or LPS plus IL-4.

ELISA assays

Assays of immunoglobulin or cytokine levels in serum or in culture supernatants were performed as described[45].

In situ hybridization

Samples of synovial tissue from patients with the clinical diagnosis of rheumatoid arthritis were collected at the time of joint arthroplasty as discarded materials. Tissue collection was approved by the Institutional Review Board. *In situ* hybridization was performed with an XBP-1-specific riboprobe as described[25,26].

Retroviral transduction of B cells

The XBP-1 complementary DNA was cloned into the retroviral vector pRV-GFP, which contains the gene for green fluorescent protein expression. A calcium-phosphate transfection was used to introduce the DNA into the Phoenix cell line as described[46]. Viral supernatants were collected after 48 h and frozen at −80 °C for later use. B cells were purified from mouse spleens using positive selection on B220+ magnetic beads as described by the manufacturer (MidiMacs, Miltenyi Biotech). B-cell purity was generally near 95%. The B cells were then activated in culture with LPS (25 μg ml^{-1}) for 24 h. The cells were spun out, mixed with 4 mg of polybrene and 1 ml of virus-containing supernatant and spun at 6,400 r.p.m. for 30 min at 32 °C. Cells were sorted for GFP expression and were grown for a further 1–4 d in culture, with or without further activating stimuli.

PCR amplification of immunoglobulin transcripts

Total RNA was isolated on day 3 or 4 from 10^7 LPS-stimulated splenocytes using the Trizol reagent (GIBCO/BRL) as per the manufacturer's instructions. We generated cDNA by using Superscript (GIBCO/BRL) according to the manufacturer's instructions. For IgM the primers used were 5′-TCCTCCTGAGCCTCTTCTAC-3′ and 5′-CCAGACATTG CTTCAGATTG-3′ (membrane) and 5′-CACACTGTACAATGTCTCCCT-3′ and 5′-AAAATGCAACATCTCACTCTG-3′ (secreted transcripts), sense and antisense, respectively. Germline IgG2b, IgG3 and mb1 transcripts were amplified with methods and primers as described[47].

Flow cytometry analysis

Single-cell suspensions from spleens were prepared as described[47]. Briefly, cells from day 4 or 5 cultures were washed in PBS with 2% FCS and stained with various antibodies conjugated with phycoerythrin (IgG2b, IgG3) and Cychrome (B220) (PharMingen). The cells were analysed with FACScalibur (BectonDickinson, Sparks) and Cellquest software, and are presented as dot plots or histograms after gating for live cells.

Immunization

Wild-type 129/Sv mice and XBP-1/RAG$^{-/-}$ chimaeric mice (10–13 weeks old) were immunized as described[48] with a single dose of 100 μg of TNP-CGG or 50 μg TNP-Ficoll (Biosearch) that had been precipitated in alum. All antigens were resuspended in saline and injected intraperitoneally.

Polyoma virus experiments

Adoptive transfer of B-cell-containing splenocyte populations of wild-type and mutant (XBP-1$^{-/-}$) mice into C57BL/SCID mice was performed as described[49]. Briefly, spleen cell suspensions were obtained by homogenizing spleens between frosted-glass microscope slides, and the erythrocytes were lysed by treatment with 0.83% ammonium chloride. After *in vitro* T-cell depletion with rat anti-mouse Thy1.2 antibody (PharMingen) and rabbit complement (Pel-Freez, Brown Deer), the number of viable cells was counted and the purity (99%) assessed by FACS. Spleens of 129/Sv and XBP-1$^{-/-}$ mice yielded comparable numbers of cells after T-cell depletion and in the 129/Sv and XBP$^{-/-}$ adoptive transfer experiments, 5×10^7 cells were injected intravenously into each SCID mouse.

Virus Ag-specific ELISA assays were done using purified VP1 PyV capsid Ag (50 ng $well^{-1}$) that was produced by recombinant baculovirus expression vectors in Sf9 insect cells and purified (a gift of R. Garcea). The serum samples were tested in duplicate using biotinylated goat anti-mouse IgM or IgG and streptavidin-HRP (Vector, Burlingame) to detect IgM or IgG, respectively.

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EXHIBIT 4

Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1

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The transcription factor X-box binding protein 1 (XBP-1) is essential for the differentiation of plasma cells and the unfolded protein response (UPR). Here we show that UPR-induced splicing of XBP-1 by the transmembrane endonuclease IRE1 is required to restore production of immunoglobulin in XBP-1$^{-/-}$ mouse B cells, providing an integral link between XBP-1, the UPR and plasma cell differentiation. Signals involved in plasma cell differentiation, specifically interleukin-4, control the transcription of XBP-1, whereas its post-transcriptional processing is dependent on synthesis of immunoglobulins during B cell differentiation. We also show that XBP-1 is involved in controlling the production of interleukin-6, a cytokine that is essential for plasma cell survival. Thus, signals upstream and downstream of XBP-1 integrate plasma cell differentiation with the UPR.

During a primary humoral immune response, naive B cells interact with cognate antigens in the peripheral lymphoid organs. Coupled with appropriate costimulatory signals, this interaction leads to a complex series of events that ultimately results in the development of both antibody-secreting plasma cells and antigen-specific memory B cells. Plasma cells are terminally differentiated effector cells that secrete large amounts of immunoglobulin (Ig) proteins. To handle this output, the plasma cell must greatly increase its secretory machinery. This increase can be seen by histological assessment, which shows that most of the cytoplasm of a terminally differentiated B cell comprises closely spaced cisternae of rough endoplasmic reticulum (ER) and secretory granules. The molecular mechanisms used by a plasma cell to accommodate this vast increase in Ig protein and to support terminal differentiation are largely unknown.

The unfolded protein response (UPR) was first described in studies that examined the proximal signals responsible for inducing the stress proteins GRP78 (also known as BiP) and GRP94. Overexpression of misfolded proteins in the ER was found to be a primary signal for the increased production of these molecular chaperones[1]. Subsequently it became clear that the UPR exists in all eukaryotes. The molecular basis of this highly coordinated response strongly suggests that it is essential for the folding, processing, export and degradation of all proteins emanating from the ER during stressed and normal conditions.

Delineation of the signaling pathway involved has been carried out mainly in the budding yeast *Saccharomyces cerevisiae*[2]. The most proximal signal from the lumen of the ER is received by a transmembrane endoribonuclease and kinase called Ire1p[3,4]. By mechanisms that are currently unclear, Ire1p senses the overabundance of unfolded proteins in the lumen of the ER. The oligomerization of this kinase leads to the activation of a C-terminal endoribonuclease by *trans*-autophosphorylation of its cytoplasmic domains[5,6]. The only known substrate for Ire1p is the transcription factor Hac1. In yeast, *HAC1* mRNA is not translated under normal conditions owing to the presence of an intron that can attenuate translation by an unusual base-pairing mechanism[7,8]. On induction of the UPR, the intron is removed by the site-specific endoribonuclease activity of Ire1p. On religation by the transfer RNA ligase Rlg1p, *HAC1*^{i} mRNA is translated into Hac1p, a potent transcriptional activator of UPR genes[9,10].

Studies examining the mammalian UPR have identified a more complex response that has additional components but shares the basic framework found in the yeast stress response. At least two Ire1p homologs in mammalian cells have been described: IRE1α, which is expressed ubiquitously[11], and IRE1β, which is present only in the gut epithelium[12]. A second ER transmembrane component, protein kinase R–ER-related kinase (PERK), has been identified independently. This ER-localized kinase is a member of the eIF2α family of kinases. Phosphorylation of eIF2α is involved in attenuating translation in response to ER stress[13,14]. A third ER transmembrane component of the mammalian UPR, named ATF6, has been identified using a conserved region of 19 base pairs (bp) of the human GRP78 promoter as bait in a yeast one-hybrid approach[15]. ATF6, a basic leucine zipper (bZIP) transcription factor, is expressed constitutively in its inactive form in the membrane of the ER. Activation in response to ER stress results in proteolytic cleavage of its N-terminal cytoplasmic domain to produce a transcriptional activator that can induce genes involved in the UPR.

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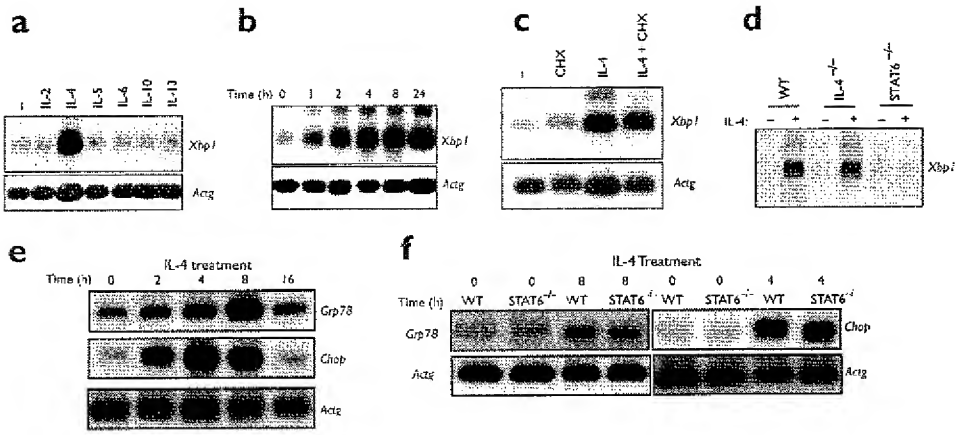

Figure 1. Induction of *Xbp1* mRNA in naive B cells by IL-4. (a) B220$^+$ splenic B cells were cultured in the presence of 20 ng/ml of the indicated cytokines for 24 h. Abundance of *Xbp1* mRNA was determined by RNA hybridization analysis and *Actg* (γ-actin) mRNA was measured as a control. (**b,c**) Splenic B cells were stimulated with recombinant IL-4 for 4 h in the absence or presence of 20 μg/ml cycloheximide (CHX), which was added to the cells 30 min before stimulation with IL-4. (**d**) Splenic B cells were taken from wild-type, IL-4$^{-/-}$ or STAT6$^{-/-}$ BALB/c mice and stimulated with recombinant IL-4 for 16 h. The abundance of *Xbp1* mRNA was determined as in **a**. (**e**) Splenic B cells were treated with recombinant IL-4 for the indicated times, and the abundance of *Grp78* and *Chop* mRNA was determined by RNA hybridization analysis. (**f**) Splenic B cells were taken from either wild-type or STAT6$^{-/-}$ mice and stimulated with recombinant IL-4 for up to 8 h. The abundance of *Grp78* and *Chop* mRNA was determined as in **e**. Results are representative of three separate experiments.

The missing element in these pathways was the substrate for IRE1α, or the mammalian homolog of Hac1p. The yeast one-hybrid screen that yielded ATF6 also identified the binding of another bZIP transcription factor named XBP-1; however, its role in the UPR was unknown. Three independent groups working in both mammalian and *Caenorhabditis elegans* model systems have shown that *XBP1* mRNA undergoes IRE1-dependent splicing in response to ER stress[16–18]. This splicing event in mammalian cells removes a 26-bp fragment, inducing a frameshift of the mRNA transcript. Translation of the new reading frame results in the conversion of XBP-1 from an unspliced form of 267 amino acids to a spliced form of 371 amino acids that comprises the original N-terminal DNA binding domain plus an additional transactivation domain in the C terminus.

XBP-1 is a member of the CREB/ATF family of transcription factors and was first isolated in our laboratory through its ability to bind a cyclic AMP response element (CRE) sequence in the gene encoding the major histocompatibility complex (MHC) class II molecule DRα. In adult tissues it is expressed ubiquitously, but in fetal tissues it is expressed preferentially in exocrine glands, osteoblasts, chondroblasts and liver[19,20]. XBP-1 is essential for the differentiation of hepatocytes, because XBP-1–deficient embryos die *in utero* from severe liver hypoplasia and a resulting fatal anemia[21]. In B cell lines representing lineage development from pre-pro-B cells to plasma cells, XBP-1 is expressed most highly in plasma cell lines. Consistent with these data, *in situ* hybridization has shown that XBP-1 is highly expressed in plasma cells in joint synovium from individuals affected with rheumatoid arthritis. Transient expression studies show that the promoter of XBP-1 is strongly downregulated by the transcription factor PAX5 (also known as BSAP), which may account for the high expression of XBP-1 seen in some plasma cell lines at a stage of development when PAX5 is no longer present[22].

XBP-1 is required for generating plasma cells[23]. When introduced into B-lineage cells, XBP-1 initiates plasma cell differentiation. XBP-1$^{-/-}$RAG$^{-/-}$ lymphoid chimeras possess normal numbers of activated B lymphocytes that proliferate, secrete cytokines and form normal germinal centers; however, they secrete very little immunoglobulin (Ig) of any isotype either basally or in response to immunization with T-independent or T-dependent antigens and also do not control infection with the B cell–dependent polyoma virus. These profound defects have been explained by the absence of plasma cells in XBP-1–deficient lymphoid tissues. XBP-1 is the first transcription factor shown to be selectively and specifically required for the terminal differentiation of B lymphocytes to plasma cells[23].

Although the role of the UPR in cells undergoing stress from environmental stimuli or drugs that disrupt ER homeostasis has become clearer, the function of the UPR in cell differentiation has not been described. Here we have investigated the relationship between plasma cell differentiation, XBP-1 and the UPR and show that this signaling system is essential for plasma cell differentiation and is controlled by the signals that regulate commitment to the B cell lineage. The expression of mouse *Xbp1* and other genes involved in the UPR is controlled by interleukin-4 (IL-4), a cytokine that is crucial for B cell survival, whereas production of the spliced *Xbp1* transcript and the spliced XBP-1 protein occurs during activated B cell progression to the plasma cell stage. Production of the spliced form of XBP-1 protein is dependent on synthesis of Ig during B cell differentiation. Only the spliced form of XBP-1 protein can restore secretion of Ig in XBP-1–null primary B cells. In addition, XBP-1 has a previously unsuspected role in controlling plasma cell differentiation because the spliced form of XBP-1 induces the production of IL-6, a cytokine that is essential for plasma cell differentiation and myeloma cell growth.

Results

Rapid induction of *Xbp1* by IL-4 in primary B cells

Because XBP-1 is required for the terminal differentiation of B cells, we first identified the stimuli that regulate expression of *Xbp1*. Several cytokines (IL-2, IL-4, IL-5, IL-6, IL-10 and IL-13) have been implicated in plasma cell differentiation both *in vitro* and *in vivo*[24]; therefore, we tested whether any of these cytokines could upregulate *Xbp1* mRNA in mouse B cells. Purified splenic B cells were cultured for 24 h in the presence or absence of various cytokines, and expression of *Xbp1* was determined by RNA hybridization analysis. IL-4 alone considerably upregulated *Xbp1* mRNA 24 h after cytokine treatment, whereas the other cytokines tested were inert as compared with untreated cells (**Fig. 1a**). Inclusion of the latter cytokines in IL-4–treated cultures did not result in a further increase of *Xbp1* transcript beyond that seen with IL-4 alone (data not shown).

To define more precisely the kinetics of this upregulation, purified B cell cultures were treated with IL-4 and assayed at intervals up to 24 h (time points after 24 h were not tested because of low cell viability). We found that *Xbp1* transcripts increased rapidly in response to

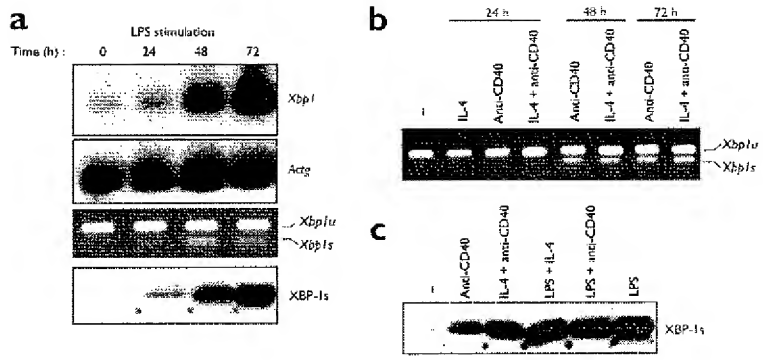

Figure 2. IRE1-mediated splicing of *Xbp1* during plasma cell differentiation. (**a**) Splenic B cells were cultured in the presence of 20 μg/ml of LPS for 3 d. Total RNAs were prepared at the indicated times and the abundance of *Xbp1* mRNA was determined by RNA hybridization analysis with *Actg* mRNA measured as a control (top two panels). RT-PCR analysis was done with a primer set flanking the spliced-out region in *Xbp1s* mRNA. PCR products were resolved on 3% agarose gels to separate unspliced (*Xbp1u*) and spliced (*Xbp1s*) *Xbp1* mRNAs (third panel). XBP-1s protein was measured by immunoblot analysis (bottom panel). (**b**) Splenic B cells were stimulated with IL-4, anti-CD40 or both for the indicated times. RT-PCR analysis was done as in **a**. (**c**) Immunoblot analysis of Xbp1s protein in cells stimulated as indicated for 72 h. Results are representative of three separate experiments.

IL-4: they were detected within 1 h of IL-4 treatment and peaked at about 8 h (**Fig. 1b**). To determine whether this induction required the synthesis of new protein, we treated cultures with IL-4 in the presence or absence of cycloheximide, an inhibitor of protein synthesis. Cycloheximide did not affect the upregulation of *Xbp1* mRNA in response to IL-4 (**Fig. 1c**), which suggested that the rapid upregulation of *Xbp1* mRNA in IL-4–treated B cells might depend on its direct transcriptional activation by the IL-4 receptor–linked signaling protein, STAT6. To test this hypothesis, B cells from wild type, IL-4$^{-/-}$ and STAT6$^{-/-}$ mice were treated with IL-4. Control B cells and IL-4$^{-/-}$ B cells stimulated as described above for 18 h considerably upregulated *Xbp1* mRNA, whereas STAT6$^{-/-}$ B cells did not induce *Xbp1* mRNA on treatment with IL-4 (**Fig. 1d**).

To determine whether the expression of other components of the UPR pathway also might be induced by IL-4, we examined IL-4–treated purified B cells for expression of the ER chaperone GRP78 and the UPR-associated transcription factor gene CHOP (also known as Gadd153). IL-4 treatment resulted in a rapid induction of *Grp78* mRNA (within 2 h), with kinetics comparable to those of *Xbp1* (peaking by 8 h). *Chop* mRNA was induced rapidly within 2 h of IL-4 treatment and reached maximal amounts by 4 h. In contrast to *Xbp1*, both the *Grp78* and *Chop* transcripts returned to baseline 16 h after treatment with IL-4 (**Fig. 1e**). B cells from wild-type and STAT6$^{-/-}$ mice treated with IL-4 did not show differences in the induction of *Grp78* and *Chop* mRNA (**Fig. 1f**). Thus, *Xbp1* mRNA in B cells is controlled by IL-4 in a STAT6-dependent manner. IL-4 also affects the regulation of GRP78 and CHOP, which are both elements of the UPR, but in a STAT6-independent manner.

XBP-1 splicing correlates with plasma cell differentiation

The UPR is induced in cells that detect irregular amounts of unfolded or unassembled protein in the lumen of the ER. Before its secretion from activated B cells, the increased load of Ig in the ER could be an effective signal to activate IRE1α and the subsequent splicing of XBP-1. To investigate this possibility, we examined the ability of various stimuli to induce splicing of XBP-1. Stimulation through the CD40 receptor plus treatment with cytokines or mitogens such as lipopolysaccharide (LPS) induced activation and differentiation of mouse B cells. Both anti-CD40 and LPS have been found to upregulate *Xbp1* mRNA transcripts in purified mouse B cells[23] (**Fig. 2a**), but it was not previously established whether these transcripts encode the unspliced or spliced form of XBP-1.

We carried out polymerase chain reaction with reverse transcription (RT-PCR) analysis using mRNA from purified mouse B cells treated *in vitro* with LPS, anti-CD40 or anti-IgM with and without IL-4. Primer at positions 410 and 580 of mouse *Xbp1* were used to amplify the region encompassing the splice junction. RT-PCR analysis from untreated cells and from all groups treated for 24 h detected a predominant amplified fragment of 171 bp corresponding to unspliced *Xbp1* mRNA. The same analysis on samples treated for 48 and 72 h with LPS and anti-CD40 plus IL-4 detected the unspliced band of 171 bp and an additional band of 145 bp corresponding to the spliced form of *Xbp1* mRNA, which lacks 26 bp in this region. Spliced *Xbp1* mRNA was also detected 48 and 72 h after treatment with only anti-CD40 (**Fig. 2a,b**). Consistent with its inability to induce differentiation, stimulation through the B cell receptor alone did not induce splicing (data not shown). IL-4 also did not induce splicing after 24 h of treatment (**Fig. 2b**). These results confirm earlier reports of the production of XBP-1 spliced protein on LPS treatment[18,25] and extend it to implicate the physiologically relevant signaling pathway, the CD40 pathway, in the splicing event.

Although anti-CD40 could induce the production of the spliced *Xbp1* transcript, the production of maximal amounts of spliced XBP-1 protein required stimulation with both IL-4 and CD40, similar to what is required for producing Ig from B cells (**Fig. 2c**). Similarly, LPS treatment, which can by itself promote Ig production and plasma cell differentiation, was competent to produce substantial amounts of spliced XBP-1 protein in the absence of other stimuli (**Fig. 2a**). Thus, the ability of a given stimulus to affect XBP-1 splicing correlates with its ability to promote plasma cell differentiation.

XBP-1 splicing occurs in terminal B cell differentiation

To investigate the regulation of XBP-1 splicing in terminal B cell differentiation, we used the BCL1 mouse cell line model of plasma cell differentiation. On stimulation with IL-2 and IL-5, this mature B cell line differentiates into an early plasma cell state[26]. BCL1 cells express basal amounts of *Xbp1* mRNA that do not increase on stimulation with IL-2 and IL-5 (ref. 23), but it is not known whether these stimuli provoke the splicing event.

To investigate whether XBP-1 splicing occurs during this differentiation process, we treated BCL1 cells with IL-2 and IL-5. Differentiation was indicated by an increase in the production of Ig (**Fig. 3a**) and an increase in the forward *versus* side scatter parameters (data not shown). RNA hybridization analysis also detected rapid upregulation of *Blimp1* mRNA, with a concomitant repression of *Myc* mRNA, consistent with plasma cell differentiation[27,28] (**Fig. 3b**). We carried out RT-PCR analysis using mRNA from untreated BCL1 cells or cells treated with IL-2 and IL-5, and primers to amplify the region of mouse *Xbp1* that encompasses the splice junction as described above. RT-PCR analysis from untreated cells detected an amplified fragment of 171 bp corresponding to unspliced mRNA. By contrast, the same analysis of mRNA from treated cells at 24 and 48 h detected both the unspliced (171 bp) and spliced (145 bp) fragments of the *Xbp1* mRNA (**Fig. 3c**).

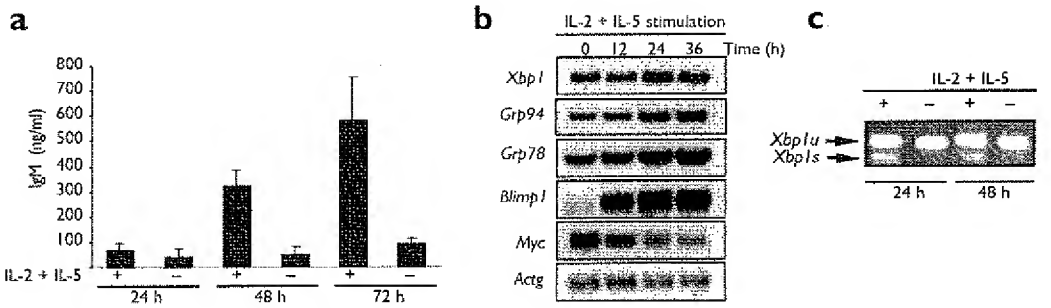

Figure 3. *Xbp1* splicing and the UPR in BCL1 terminal differentiation. (**a**) Production of IgM was measured by ELISA assay of the culture supernatants of BCL1 cells stimulated with IL-2 and IL-5 (20 ng/ml each) and control unstimulated cells for the indicated times. Experiments were done at least three times and the s.d. is indicated. (**b**) RNA hybridization analysis containing 7 μg of total RNA from BCL1 cells stimulated with IL-2 and IL-5 (20 ng/ml each) for 12, 24 and 36 h. (**c**) Total RNA from BCL1 cells either unstimulated or stimulated with IL-2 and IL-5 (20 ng/ml each) for 24 and 48 h was used for RT-PCR analysis. Primers spanning the splice junction in murine *Xbp1* were used to amplify products of unspliced and spliced mRNA. PCR products were separated by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining. Results are representative of three separate experiments.

Overexpression of the spliced form of XBP-1, but not the unspliced form, results in substantial induction of UPR reporter constructs in HeLa cells[17]. To establish a link among the splicing event, plasma cell differentiation and the UPR, we examined induction of the UPR targets GRP78 and GRP94 during the differentiation of BCL1 cells. Terminal differentiation of BCL1 cells was accompanied by substantial upregulation of *Grp94* and *Grp78* mRNA, an induction that correlated with the splicing of XBP-1 (**Fig. 3b**). These data are consistent with the idea that induction of the UPR and subsequent transcriptional activation by the spliced form of XBP-1 are involved in the terminal differentiation of B cells.

XBP-1 splicing depends on IgM heavy chain production

It is unclear whether the activity of IRE1 and subsequent splicing of XBP-1 is induced by a UPR-dependent (for example, translated Ig) or a UPR-independent mechanism during plasma cell differentiation. Resolving this issue is important because it will clarify whether plasma cell differentiation requires the UPR or not. The kinetics of XBP-1 splicing, as shown above, correlates with secretion of Ig. These observations support a role for Ig protein in XBP-1 splicing during B cell differentiation but do not provide definitive evidence. To address this issue, we used two approaches.

First, we treated LPS-stimulated primary B cells with a low concentration of cycloheximide (50 ng/ml) to inhibit protein synthesis partially. Splicing of XBP-1 did not occur, thus supporting an UPR-dependent mechanism for IRE1 endoribonuclease activity (data not shown). A similar low dose of cycloheximide is nontoxic in LPS-stimulated primary B cells but does inhibit proliferation[20]. Thus, the lack of XBP-1 splicing might be due to a general cessation in the cell cycle rather than to a specific blockade of the UPR.

Second, we examined XBP-1 splicing in a strain of mice in which the B1-8 heavy chain variable region gene is flanked by *loxP* sites (floxed)[30], enabling it to be deleted through recombination mediated by Cre recombinase. B cells from these mice develop normally *in vivo*, but on activation of Cre *ex vivo* B1-8$^{f/+}$ B cells do not produce Ig heavy chains. We isolated splenic B cells from these mice and, just before activation, deleted the Ig heavy chain *ex vivo* by transducing the cells with Tat-Cre recombinase[31]. The cells were cultured for 48 h in the presence of LPS and then sorted according to their surface IgM (sIgM). Intracellular analysis of the sIgM$^-$ population showed a complete absence of IgM heavy chain (**Fig. 4a**).

We analyzed the purified populations for spliced XBP-1 protein. The sIgM$^-$ population showed a greatly reduced amount of spliced XBP-1 as compared with the control population of stimulated B cells (sIgM$^+$; **Fig. 4b**). Thus, in the absence of IgM, the production of spliced XBP-1 was strongly diminished, providing evidence that during B cell differentiation accumulation of translated Ig heavy chains induces IRE1 activity. These data indicate that the UPR may be an important component of plasma cell differentiation.

Overexpression of XBP-1 enhances IgM secretion

Ectopic expression of XBP-1 in BCL1 cells increases the quantity of membrane CD138 (ref. 23). To determine the effect of unspliced and spliced forms of XBP-1 in the production of IgM, we generated two bicistronic retroviral vectors: one expressing both the unspliced and spliced variants of mouse XBP-1 plus green fluorescent protein (GFP), and the other encoding only the spliced variant of the XBP-1 protein plus GFP.

Expression of the first vector, Xbp1u/s, produced proteins of 267 amino acids (33 kDa, unspliced) and 371 amino acids (54 kDa, spliced), respectively. Expression of the second vector, Xbp1s, produced only a protein containing 371 amino acids. Overexpression in NIH3T3 fibroblasts by transient transfection of Xbp1u/s and Xbp1s

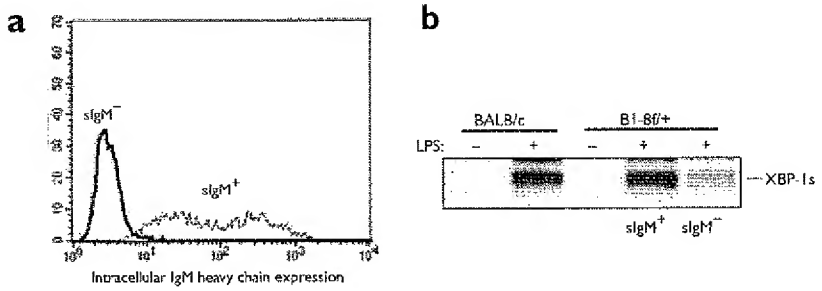

Figure 4. Production of spliced XBP-1 depends on the IgM heavy chain. (**a**) B1-8$^{f/+}$ B cells transduced with Cre were stimulated for 40–48 h with 20 μg/ml of LPS. Cells were stained for sIgM with PE-conjugated anti-IgM, and subpopulations of sIgM$^+$ and sIgM$^-$ were analyzed for intracellular IgM by staining with FITC-conjugated anti-IgM. (**b**) Immunoblot analysis of Xbp1s protein in subpopulations of sIgM$^+$ and sIgM$^-$ cells from stimulated B1-8$^{f/+}$ B cells transduced with Cre recombinase.

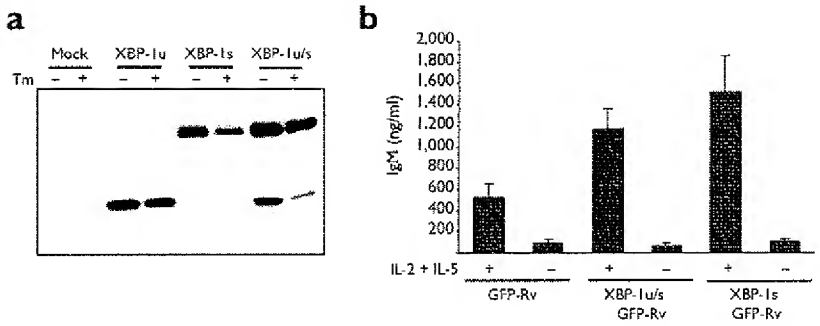

Figure 5. Ectopic expression of spliced XBP-1 enhances IgM secretion. (a) Total cell lysates from transfected NIH3T3 cells unstimulated and stimulated with tunicamycin (Tm) were used for immunoblot analysis. (b) BCL1 cells were transduced with retroviruses encoding GFP alone (GFP-Rv) or GFP plus *Xbp1u/s* or *Xbp1s*. After 30 h of transduction, the cells were sorted for GFP and incubated in the presence or absence of IL-2 and IL-5 (20 ng/ml each) for 72 h. Production of IgM was analyzed by ELISA assays of the culture supernatants of unstimulated and stimulated BCL1 cells. Experiments were done at least three times and the s.d. is indicated.

cDNA confirmed the appropriate production of the unspliced and spliced forms of XBP-1, as assessed by immunoblot analysis (**Fig. 5a**).

We transduced these vectors into the BCL1 cell line and sorted for GFP expression to generate stable populations of cells expressing control vector, Xbp1u/s and Xbp1s. The cells were treated with IL-2 and IL-5, or left untreated, and then assayed for IgM secretion. In GFP-positive BCL1 cells that were treated with IL-2 and IL-5 for 3 d, expression of Xbp1u/s increased the secretion of IgM roughly threefold as compared with cells expressing control vector. Similarly, expression of Xbp1s in cytokine-treated cells enhanced IgM secretion roughly fourfold as compared with control cells. In GFP-positive BCL1 cells that were untreated with cytokine, neither Xbp1u/s nor Xbp1s could induce the secretion of IgM (**Fig. 5b**). These data show that the spliced variant of XBP-1 can drive the production and secretion of Ig in the presence of stimuli required for terminal plasma cell differentiation.

Spliced XBP-1 restores Ig production in XBP-1$^{-/-}$ B cells

After *in vitro* stimulation with LPS, XBP-1–deficient B cells produce very little Ig as compared with wild-type B cells, but ectopic expression of a cDNA encoding both the unspliced and spliced forms of XBP-1 in the XBP-1–deficient B cells partially restores Ig secretion[23].

To examine the relative contributions of the unspliced and spliced forms of XBP-1 in Ig production in primary cells, we produced a mutant of *Xbp1*, similar to those described for yeast and human *XBP1* mRNA, that could not be spliced by IRE1α[32,33]. Ire1p-dependent splicing of the 3′ splice site of yeast *HAC1* mRNA is dependent on positions –3, –1, +3 and +4 in the seven-nucleotide loop structure that is targeted for splicing. Mutations of any of these four crucial sites results in transcripts that cannot be spliced in response to tunicamycin treatment, which induces the UPR through inhibition of glycosylation. Accordingly, we created point mutations at positions –1 and +3 in the loop structure of mouse *Xbp1* in a vector that we designated Xbp1u. Overexpression of this vector resulted in the production of a single protein of 33 kDa, confirming exclusive production of the unspliced XBP-1 protein (**Fig. 5a**).

We compared the effects of these three different forms of XBP-1 on Ig secretion in wild-type and XBP-1$^{-/-}$ B cells. *In vitro*–activated splenic B cells were transduced using retroviruses expressing bicistronic mRNA encoding Xbp1u/s, Xbp1s or Xbp-1u plus GFP or control GFP alone. After cell sorting, GFP-positive cells were stimulated with LPS and assayed for Ig production. As above, XBP-1$^{-/-}$ cells were substantially impaired in their production of IgM, as wild-type cells expressing control GFP expressed roughly 20 times more IgM than did similar GFP-vector transduced XBP-1$^{-/-}$ cells. Yet expression of Xbp1u/s or Xbp1s increased the secretion of IgM in XBP-1$^{-/-}$ B cells roughly tenfold as compared with control cells. By contrast, expression of Xbp1u did not increase IgM secretion in XBP-1$^{-/-}$ B cells (**Fig. 6a**).

LPS is known to induce class switching from IgM to IgG_{2b} subclasses on *in vitro* stimulation of B cells23. We previously reported that production of IgG_{2b} by stimulation with LPS is greatly reduced in XBP-1$^{-/-}$ B cells[23]. We therefore examined the ability of the two variants of XBP-1 to restore IgG_{2b} production in XBP-1$^{-/-}$ B cells. Wild-type cells expressing control GFP expressed five times more IgG_{2b} than did similar control transduced XBP-1$^{-/-}$ cells. Expression of Xbp1u/s or Xbp1s retroviruses increased the secretion of IgG_{2b} in XBP-1$^{-/-}$ B cells between two- and fourfold as compared with control cells. By contrast, the expression of Xbp1u was unable to increase IgG secretion in XBP-1$^{-/-}$ B cells (**Fig. 6b**). We conclude that the spliced form of XBP-1 but not the unspliced form is responsible for, and indeed required for, the production of secreted Ig in normal B lymphocytes. Thus, the signaling system set in motion by the UPR is required for B cell differentiation.

Spliced XBP-1 induces IL-6 secretion

IL-6 can drive purified B cells into Ig-secreting plasma cells and function as an important growth factor for malignant plasma cells[34,35].

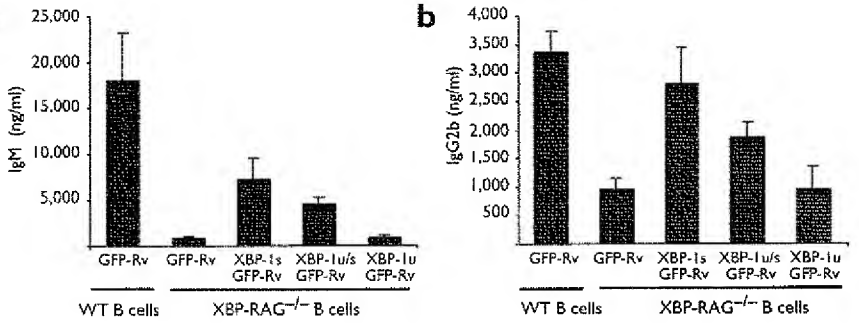

Figure 6. Spliced XBP-1 restores Ig production in XBP-1$^{-/-}$ B cells. (a,b) Purified B cells from wild-type and XBP-1$^{-/-}$ mice were activated in culture with 10 μg/ml of LPS and 5 μg/ml of $F(ab')_2$ anti-IgM for 24 h and then transduced with retroviruses encoding GFP alone (GFP-Rv) or GFP plus *Xbp1u/s*, *Xbp1s* or *Xbp1u*. The cells were incubated for 24–36 h at 37 °C, sorted by flow cytometry for GFP$^+$ cells and then returned to culture with stimulation with 10 μg/ml of LPS for 72 h. Production of IgM (a) and IgG2b (b) in the culture supernatants of the indicated stimulated B cells was analyzed by ELISA. Experiments were done at least three times and the s.d. is indicated.

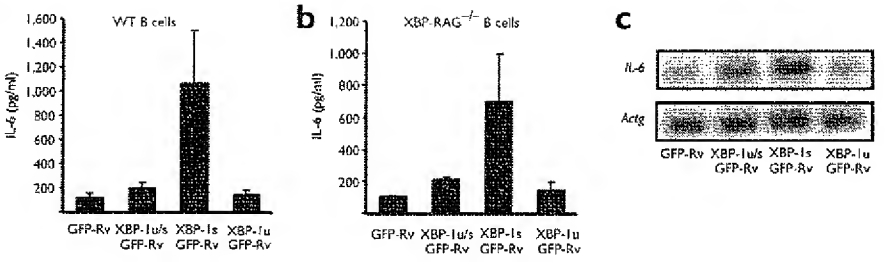

Figure 7. Spliced XBP-1 induces IL-6. (**a**,**b**) Purified B cells from wild-type (**a**) and XBP-1$^{-/-}$ (**b**) mice were activated in culture with 10 μg/ml of LPS and 5 μg/ml of F(ab')$_2$ anti-IgM for 24 h and then transduced with retroviruses encoding GFP alone (GFP-Rv) or GFP plus Xbp1u/s, Xbp1s or Xbp1u. The cells were incubated for 24–36 h at 37 °C, sorted by flow cytometry for GFP$^+$ cells and then returned to culture with stimulation by 10 μg/ml of LPS for 72 h. Production of cytokine was analyzed by ELISA assay of the culture supernatants of stimulated B cells. Experiments were done at least three times and the s.d. is indicated. (**c**) Total RNAs were prepared from the stimulated B cells described in a and the abundance of *Il6* mRNA was determined by RNA hybridization analysis with *Actg* mRNA measured as a control.

XBP-1 is induced by IL-6 treatment and implicated in the proliferation of malignant plasma cells[36]. Here, however, we found no role for IL-6 in upregulating *Xbp1* transcripts or in mediating splicing of *Xbp1* RNA in mature B cells (**Fig. 1a** and data not shown). Given the known role of IL-6 in plasma cell differentiation, we considered whether IL-6 might function downstream of XBP-1. We therefore examined whether XBP-1 induces the production of IL-6 in wild-type and XBP-1$^{-/-}$ primary B cells.

In vitro–activated mouse splenic B cells were transduced using retroviruses expressing Xbp1u/s, Xbp1s, Xbp1u and control GFP as above. After cell sorting, GFP-positive cells were stimulated with LPS and assayed for cytokine production after 72 h. Wild-type and XBP-1$^{-/-}$ cells expressing control GFP or Xbp1u retroviruses expressed moderate amounts of IL-6, similar to previous studies[37]. Expression of Xbp1u/s increased IL-6 secretion roughly twofold as compared with cells expressing control vector. Expression of Xbp1s increased the secretion of IL-6 in wild-type and XBP-1$^{-/-}$ B cells about tenfold and sevenfold, respectively, as compared with cells expressing control vector (**Fig. 7a,b**).

RNA hybridization analysis of mRNA from cells transduced with Xbp1s detected a roughly fivefold increase in *Il-6* gene expression as compared with controls (**Fig. 7c**). The expression of other cytokines, including IL-2, IL-4, IL-5 and IL-10, was unaffected. These data suggest that, in addition to its role in the UPR, the spliced form of XBP-1 also regulates the expression of the plasma cell growth factor IL-6.

Discussion

So far, XBP-1 is the only transcription factor that has been found to be essential for plasma cell differentiation. Given its newly defined role in the UPR, we speculated that this signaling pathway also might be required for terminal B cell differentiation. Our experiments support this model because signals that induce plasma cell differentiation and the UPR cooperate to induce both expression of *Xbp1* mRNA and splicing of the resulting transcript. The specific requirement for the spliced form of XBP-1 to restore production of Ig in XBP-1$^{-/-}$ B cells provides compelling evidence that plasma cell differentiation is dependent on UPR-induced splicing of XBP-1 by IRE1α. XBP-1 also has an additional role in controlling plasma cell differentiation—that of regulating the production of IL-6—that complements its function in the UPR.

IL-4 is a multifunctional cytokine produced by T cells, mast cells and basophils that induces the activation and proliferation of B cells, stimulates Ig class switching to IgG$_1$ and IgE, and prevents apoptosis[38]. Its importance in early B cell activation suggests that IL-4 also may be involved in plasma cell differentiation, an idea that is supported by our finding that IL-4 is the only cytokine that controls expression of *Xbp1* in the mature B cell. The generation of maximal amounts of XBP-1 protein also required IL-4. An activated B cell must negotiate a delicate balance of apoptosis, proliferation and differentiation to become a plasma cell. It has been suggested that IL-4 promotes B cell activation through the reduction of inhibitory receptors on activated B cells, indicating a key role for this cytokine in promoting cell survival[39].

The importance of cell survival *versus* apoptosis in plasma cell differentiation is highlighted by reports on the transcription factor Blimp1 (also called PRDF1-BF1), which is known to drive the differentiation of B cells to plasma cells. This zinc-finger protein is expressed specifically in mature B cells and plasma cells, and overexpression of Blimp1 in the BCL1 cell line causes the appearance of an early plasma cell phenotype, including J-chain expression and Ig secretion[27]. Blimp1 promotes plasma cell generation partly through the repression of Myc, thereby allowing the B cell to exit the cell cycle and undergo terminal differentiation[28]. So far, however, we have not found a function of XBP-1 in controlling Myc expression, and cell survival or apoptosis is not markedly altered in B cells lacking XBP-1 (N. N. Iwakoshi and A.-H. Lee, unpublished data), suggesting that IL-4–induced XBP-1 has alternative, still undefined functions in early B cell activation. Indeed, expression of Blimp1 is normal in XBP-1$^{-/-}$ B cells, so it is likely that these two transcription factors have complementary rather than overlapping functions in lineage commitment.

It has been proposed that the induction of ER chaperones can be controlled by extracellular stimuli (growth factors) in addition to pathways emanating from a stressed ER[40]. A study using the CH12 B cell lymphoma model has suggested that expression of XBP-1, GRP78 and GRP94 is initiated before the increase in translation of Ig chains caused by LPS-induced differentiation. This has led to speculation that there must be an early signal in B cell differentiation that initiates the UPR before the translation of Ig. Our data show that IL-4 may be that signal because it rapidly induced the expression of XBP-1, GRP78 and CHOP within 2 h in freshly isolated primary B cells. We cannot rule out the possibility that IL-4–induced synthesis of Ig in activated B cells contaminating our population of freshly isolated resting B cells was responsible for the induction of these UPR markers; however, the marked dependency on STAT6 of the immediate and early induction of *Xbp1* mRNA argues against ER-dependent transcriptional control of XBP-1 by IL-4. These observations indicate that IL-4 may initiate the UPR early in B cell differentiation by upregulating XBP-1, and possibly GRP78 and CHOP, to help ensure efficient processing of subsequently produced Ig.

In contrast to IL-4, which functions in early B cell activation, the cytokine IL-6 was originally described to function during late-stage plasma cell differentiation and as a growth factor for myeloma cells[41]. Overexpression of IL-6 in transgenic mice leads to plasmacytosis and, in certain background strains, to frank plasmacytoma, whereas

IL-6–deficient mice show severe decreases in IgG and IgA secretion[41–43]. We did not find evidence for a role for IL-6 in regulating *Xbp1* transcript abundance or splicing in the mature B cells; however, IL-6 upregulates *Xbp1* mRNA in myeloma cell lines[36]. It is tempting to speculate that IL-4, because of its effects early in B cell differentiation, sustains XBP-1 expression early during differentiation, whereas IL-6, which is thought to act after germinal center switching takes place, maintains expression of XBP-1 in the later stages of plasma cell growth and survival. The sequential combination of these and possibly other unidentified factors would ensure high levels of *Xbp1* mRNA during plasma cell differentiation. In this regard, it should be noted that neither IL-4–deficient nor IL-6–deficient mice show general defects in the plasma cell compartment. We therefore predict that other factors also control XBP-1 expression *in vivo*, although so far we have not identified them.

In both LPS-stimulated B cells and BCL1 cells treated with IL-2 and IL-5, the kinetics of splicing was correlated with the production or secretion of Ig. This is consistent with the idea that an accumulation of unfolded Ig in activated B cells activates the IRE1α to induce splicing of XBP-1. Stimulation through the CD40 receptor alone induced splicing in the absence of high Ig production; however, the production of maximal amounts of spliced XBP-1 protein required stimulation with both IL-4 and CD40, similar to the requirements for Ig production in B cells.

To address directly whether production of the spliced form of XBP-1 is induced by Ig biosynthesis during B cell differentiation by an UPR-dependent mechanism, we compared the amount of XBP-1 spliced protein in LPS-activated B1-8$^{f/-}$ B cells to that in control B cells. In the absence of the Ig heavy chain, splicing of XBP-1 was strongly diminished, providing evidence that translated Ig heavy chains induce IRE1 activity during B cell differentiation. These data provide direct evidence that biosynthesis of Ig is involved in producing the spliced form of XBP-1 protein and, more importantly, that the UPR is an essential component of plasma cell differentiation.

Other studies in yeast and mammalian cell lines attempting to address the activation of IRE1 support an ER-dependent mechanism. In this model, GRP78 binds to the luminal domain of IRE1, thereby inhibiting its activation. On protein biosynthesis, the unfolded proteins in the ER compete for chaperones such as GRP78, thus allowing the dimerization and subsequent activation of IRE1 (refs. 44,45). Notably, GRP78 is also known as immunoglobulin-binding protein owing to its ability to bind non-covalently bound heavy chain[46].

It should be noted that in studies where deletion and subsequent downregulation of sIgM is completed before activation, sIgM$^-$B1-8$^{f/+}$ cells show a significant decrease in rates of activation and proliferation. In our studies, however, activation is done concurrently with deletion, and the complete loss of sIgM occurs 30 h after LPS stimulation. On the basis of cell size, B cell activation is equivalent in cells carrying floxed B1-8$^{f/+}$ and unfloxed controls during the initial 40–48 h of LPS stimulation. In addition, as another measure of comparable activation, the production of Ig light chain in floxed B1-8$^{f/+}$ cells is decreased only slightly. Because Ig light chains are still upregulated, the presence of these proteins or others during stimulation with LPS may account for the small amount of spliced XBP-1 protein that is produced in activated floxed B1-8$^{f/+}$ B cells. Alternatively, direct signaling pathways that activate IRE1 may also complement UPR-dependent activation.

What then is the relationship between the role of XBP-1 in evoking the classical stress response, as determined by the induction of ER chaperone genes, and its role in inducing plasma cell differentiation? Clearly, both responses rely on the generation of spliced XBP-1 protein. Only spliced XBP-1 can reconstitute Ig secretion in Xbp1$^{-/-}$ B cells and only spliced XBP-1 is a potent transcriptional activator for ER chaperone genes[17,47]. Mammalian XBP-1 is a target of IRE1α and we have shown that, for plasma cell differentiation, XBP-1 lies downstream of IRE1α. Thus, it is essential to understand the function of IRE1α.

Currently, however, the physiological role of the mammalian IRE1 homologs in the UPR is not clearly defined. Previous data have suggested that these kinases are not essential for certain ER stress responses. Mouse embryonic fibroblasts (MEFs) deficient in IRE1α and IRE1β show a normal ER stress response, as assessed by their unimpaired transcriptional induction of several UPR chaperone genes and survival in culture[48,49]. In this regard, we have found that, similar to the phenotype of MEFs deficient in IRE1α and IRE1β, the induction of the UPR chaperone genes GRP78 and GRP94 is only slightly impaired in XBP-1$^{-/-}$ B cells (N. N. Iwakoshi and A.-H. Lee, unpublished data). We conclude that the role of XBP-1 in the UPR and its role in plasma cell differentiation do not completely overlap. Clearly, the signaling pathways that are redundant for the former (such as ATF6 and PERK) are not redundant for the latter. Taken together, our results support the idea that induction and splicing of *Xbp1* mRNA results from a collaborative effort between the UPR and the signals that induce plasma cell differentiation. It is also curious that, in contrast to *HAC1* mRNA[7], unspliced *Xbp1* mRNA in mammalian cells is translated. Given the greater complexity of the mammalian *versus* the yeast UPR, it is possible that the unspliced protein has a previously unknown function in plasma cell differentiation.

XBP-1 is required absolutely for plasma cell differentiation, but its role in driving the transcription of at least a subset of ER chaperone genes is redundant. This raises the possibility that XBP-1 has unidentified functions and target genes that are unrelated to the UPR. We considered that IL-6 might be one such target gene because of the importance of this cytokine both in plasma cell differentiation and transformation (in multiple myeloma), and in autoimmune diseases such as rheumatoid arthritis, mesangial glomerulonephritis and Castleman's disease. Our results support the existence of a positive feedback loop between IL-6 and XBP-1 that would ensure high quantities of both proteins during plasma cell differentiation. Whereas spliced XBP-1 protein can confer on B cells the ability to produce IL-6, a comparison of wild-type and XBP-1$^{-/-}$ B cells stimulated with LPS *in vitro* showed no significant difference in IL-6 production. Thus, XBP-1 is not essential for or required for expression of IL-6. This is not necessarily surprising as several transcription factors, C/EBP-β and NF-κB among others, regulate the gene encoding IL-6 (ref. 50).

The mechanism by which spliced XBP-1 controls IL-6 gene expression is not known but may be indirect, because we have detected very little *trans*-activation of a 1.2-kb IL-6 promoter construct by XBP-1 in preliminary studies. The existence of an XBP-1–IL-6 autoregulatory loop may explain the observation that IL-6–deficient mice show defects similar to those of XBP-1–deficient mice outside the immune system, with impaired production of acute-phase reactants and defects in hepatocyte growth[51,52]. In any event, the control of IL-6 expression by the spliced XBP-1 protein is an attractive complement to its function in the UPR.

Our experiments suggest the following scenario. As activated B cells differentiate toward the plasma cell stage, the ER is greatly expanded to accommodate the increased production and secretion of Igs. During this expansion, IRE1α-mediated splicing of XBP-1 occurs, resulting in activation of the UPR to support the high quantities of antibody production. Concurrently, plasma cell growth and survival are supported by the production of IL-6 induced by spliced XBP-1. In the absence of XBP-1, cellular UPR activity is impaired and the inefficient processing and exportation of Ig results in an accumulation of unfolded protein and consequently cell death.

This scenario is consistent with the observed phenotype of XBP-1 $^{-/-}$ RAG2 $^{-/-}$ chimeric animals which show profound impairment of plasma cell differentiation. Our results indicate that XBP-1 may be central to several signaling pathways that induce plasma cells. Small molecules that inhibit the expression or RNA splicing of XBP-1 might prove to be effective new therapeutics for treating the human disease multiple myeloma—the malignant counterpart of the plasma cell.

Methods

Mice. We obtained BALB/c and 129S6 mice from Jackson Laboratories (Bar Harbor, ME) and Taconic (Germantown, NY), respectively. B1-8^{hi} mice[30] and STAT6 $^{-/-}$ mice were used at 6–8 weeks and maintained in pathogen-free facilities in accordance with the guidelines of the Committee on Animals of Harvard Medical School.

Cell culture and cell lines. Mouse splenocytes or purified B cells (purified mature B cells were isolated from spleen and lymph nodes by magnetic CD43 depletion or B220^{+} magnetic bead selection; Miltenyi Biotech, Auburn, CA) were plated at 1×10^6 cells/ml in complete medium containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 2 mM glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin, 100 mM HEPES, 1× nonessential amino acids, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol, and stimulated with 1 μg/ml of anti-CD40 (Pharmingen, San Diego, CA) or 20 μg/ml of LPS (Sigma, St. Louis, MO). We tested the following cytokines: IL-4, IL-2, IL-5, IL-6, IL-10 and IL-13, each at 20 ng/ml (R&D Systems, Minneapolis, MN). Stimulated B cells were diluted to a cell concentration of 1×10^6 cells/ml with fresh medium every 24 h. The BCL1 (CWS13.20-3B3 ATCC CRL-1699) cell line was cultured in RPMI medium supplemented with 10% FBS, 20 μg/ml of gentamicin and 50 μM β-mercaptoethanol. To induce differentiation, we plated cells at 2×10^5 cells/ml and treated them with 20 ng/ml of recombinant mouse IL-2 and IL-5 (R&D Systems).

Plasmid construction and transient transfection. cDNAs encoding the Xbp1u/s and the Xbp1s splice variants of XBP-1 were amplified by PCR from the total RNA of untreated and tunicamycin-treated NIH3T3 cells, respectively. cDNA encoding Xbp1u was generated by PCR-based mutagenesis of the cDNA encoding Xbp1u/s, such that the two guanine residues at positions 532 and 535 were changed to adenine without changing the amino acid sequence in the Xbp1u open reading frame. Numbers are based on the sequences from GenBank (NM_013842). These cDNAs were inserted into the pcDNA3.1 plasmid between *Hin*dIII and *Apa*I sites to generate each mammalian expression plasmid. To generate retroviral vectors for Xbp1u, Xbp1u/s and Xbp1s, cDNAs were excised from pCDNA3.1-derived vectors by *Pme*I digestion and then inserted into the GFP-RV retroviral vector by blunt-end ligation[55]. We transfected NIH3T3 cells by using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer.

RNA hybridization and RT-PCR. Total RNA was isolated using TriZol (Gibco-BRL, Carlsbad, CA) or Qiashedder/Rneasy RNA purification columns (Qiagen, Valencia, CA). RNA hybridization was done as described[54]. In brief, 7–10 μg of RNA was separated by electrophoresis on 1.2% agarose, 6% formaldehyde gels, transferred onto GeneScreen membrane (NEN, Boston, MA) and covalently bound to the membrane using UV Stratalinker (Stratagene, La Jolla, CA). We used the following probes after ^{32}P labeling with the Redi-Prime labeling system (Amersham-Pharmacia, Piscataway, NJ): *Xbp1* (nucleotides 15–830 of the murine coding region), *Grp94* and *Grp78* (ref. 55; a gift of R.J. Kaufman, University of Michigan), *Blimp1* (*Nco*I-*Sca*I fragment), *Myc* cDNA, *Chop* cDNA and *Il6* cDNA. Probe hybridization was done with Ultrahyb buffer as recommended by the manufacturer (Ambion, Austin, TX). Total RNAs were used for the first-strand synthesis with the Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). PCR primers (5′-ACACGCTTGGGAATGGACAC-3′ and 5′- CCATGGGAAGATGTTCTGGG-3′) encompassing the missing sequences in Xbp1s was used for the PCR amplification with AmpliTaq Gold polymerase (Applied Biosystem, Foster City, CA). We separated PCR products by electrophoresis on a 3% agarose gel (Agarose-1000 Invitrogen) and visualized them by ethidium bromide staining.

Cre recombinase transduction. Splenic B cells were purified as described above from B1-8^{hi} floxed mice. Cells were washed with serum-free medium (Hyclone Laboratories) and resuspended at 10×10^6 cells/ml. Tat-Cre recombinase protein was expressed, purified and quantified as described[31]. We incubated the cells in serum-free medium containing 50 μg/ml of Tat-Cre for 1 h at 37 °C. After transduction, the cells were washed twice with complete RPMI, activated in culture (10^6 cells/ml) with 20 μg/ml of LPS for 40–48 h at 37 °C and then sorted by flow cytometry for sIgM^{-} and sIgM^{+} populations.

Intracellular immunoglobulin flow cytometry. We transferred 5×10^5 cells to U-bottom microwell plates in 50 μl of staining buffer (PBS containing 2% FBS and 0.1% sodium azide) and added 0.2 μg of mouse Fc block (Pharmingen) to each well. Cells were incubated for 5–10 min on ice and then surface markers (phycoerythrin (PE)-conjugated anti-IgM; R6-60.2, Pharmingen) were examined as described[25]. Blocking antibody (anti-IgM, II/41) to surface Ig was added to stained cells, which were incubated on ice in the dark for 15 min. Cells were washed twice with staining buffer, and then resuspended and incubated in 4% paraformaldehyde for 10 min at 25 °C. Cells were washed with PBS containing 5% FBS and then with saponin wash buffer (PBS containing 5% FBS and 0.1% saponin). We resuspended the cells in saponin wash buffer with 0.2 μg of mouse Fc block for 5–10 min on ice. Intracellular IgM was stained by adding 0.5 μg of fluorescein isothiocyanate (FITC)-conjugated II/41 monoclonal antibody (Pharmingen) in saponin wash buffer. After 30 min of incubation, the cells were washed twice with staining buffer and analyzed by flow cytometry[25].

Enzyme-linked immunosorbent assays (ELISAs). Assays to measure the amounts of immunoglobulins or cytokines in culture supernatants were done as described[25].

Retroviral transduction of B cells. cDNAs encoding Xbp1u/s, Xbp1s and Xbp1u were obtained and inserted into the GFP-RV retroviral vector as described above. We used Effectene transfection (Qiagen) to introduce the DNA into the Phoenix cell line according to the manufacturer's instructions[25]. Viral supernatants were collected after 48 h and frozen at −80 °C for later use. B cells were purified from mouse spleens using CD43 depletion or positive selection with B220^{+} magnetic beads according to the manufacturer's instructions (MidiMacs, Miltenyi Biotech). B cell purity was generally almost 95% as confirmed by flow cytometry. The B cells (10^6 cells/ml) were then activated in culture with 10 μg/ml of LPS and 5 μg/ml of F(ab')$_2$ anti-IgM (Southern Biotech, Birmingham, AL) for 24 h. We mixed 1 ml of activated B cells (10^6 cells/ml) with 4 μg of polybrene and 1 ml of virus-containing supernatant and spun them at 1,000*g* for 45 min at 24 °C. Cells were incubated for 24–36 h at 37 °C, and GFP-positive cells were sorted by flow cytometry and returned to culture with and without stimulation.

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Competing interests statement

The authors declare that they have no competing financial interests.

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EXHIBIT 5

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Blimp-1 Is Required for the Formation of Immunoglobulin Secreting Plasma Cells and Pre-Plasma Memory B Cells

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Summary

Blimp-1 is a transcriptional repressor able to drive the terminal differentiation of B cells into Ig-secreting plasma cells. We have created mice with a B cell-specific deletion of *prdm1*, the gene encoding Blimp-1. B cell development and the number of B cells responding to antigen appear to be normal in these mice. However, in response to either TD or TI antigen, serum Ig, short-lived plasma cells, post-GC plasma cells, and plasma cells in a memory response are virtually absent, demonstrating that Blimp-1 is required for plasmacytic differentiation and Ig secretion. In the absence of Blimp-1, CD79b$^+$B220$^-$ pre-plasma memory B cell development is also defective, providing evidence that this subset is an intermediate in plasma cell development. B cells lacking Blimp-1 cannot secrete Ig or induce μS mRNA when stimulated ex vivo. Furthermore, although *prdm1*$^{-/-}$ B cells fail to induce XBP-1, XBP-1 cannot rescue plasmacytic differentiation without Blimp-1.

Introduction

Plasma cells (PCs), the critical immune effector cells dedicated to secretion of antigen-specific immunoglobulin (Ig), develop at three distinct stages of antigen-driven B cell development (Calame, 2001). Short-lived PCs emerge in response to both thymus-independent (TI) and thymus-dependent (TD) antigens (Ho et al., 1986) in the first week after antigen exposure in extrafollicular foci of secondary lymphoid organs (Jacob et al., 1991a). These PCs have half-lives of 3–5 days (Ho et al., 1986) and secrete unmutated Ig (Jacob et al., 1991b; McHeyzer-Williams et al., 1993). TD antigens also induce a germinal center (GC) pathway involving somatic hypermutation, affinity maturation, and production of memory B cells and long-lived PCs (Jacob et al., 1991b; MacLennan and Gray, 1986; McHeyzer-Williams et al., 1991). Post-GC PCs have extended half-lives (McHeyzer-Williams and Ahmed, 1999), produce high-affinity antibody, and reside preferentially in the bone marrow (Benner et al., 1981). Memory B cells rapidly expand and differentiate into PCs in response to antigen rechallenge (MacLennan and Gray, 1986; McHeyzer-Williams et al., 2000). Memory response PCs produce high-affinity antibodies but appear to have short life spans (Driver et al., 2001; McHeyzer-Williams et al., 2000).

How each of these PCs develops in vivo remains unclear. Antigen-driven cell expansion precedes terminal differentiation in each case and cell cycle arrest is necessary for terminal PC generation (Tourigny et al., 2002). However, as opposed to B cells that become short-lived PCs, B cells that have been through the GC reaction may differ substantially in their terminal differentiation path. Two subtypes of nonsecreting memory B cells that exit the GC (Driver et al., 2001) and respond to antigen rechallenge (McHeyzer-Williams et al., 2000) have been observed. Both populations express somatically mutated BCR with evidence of affinity-based selection (McHeyzer-Williams et al., 2000) but differ in cell surface phenotype, propensity to form PCs, and proliferative capacity upon adoptive transfer (McHeyzer-Williams et al., 2000). CD138$^-$B220$^-$ memory B cells produced 25-fold more PCs, but 15-fold fewer cells were recovered after transfer compared to CD138$^-$B220$^+$ memory cells. This suggests a linear progression from post-GC CD138$^-$B220$^+$ memory B cells to CD138$^-$B220$^-$ pre-plasma memory B cells to terminally differentiated CD138$^+$B220$^{+/-}$ PCs; however, there is no genetic evidence for a developmental link between these subsets.

B lymphocyte induced maturation protein-1 (Blimp-1) was initially described as a "master regulator" of plasma cell differentiation (Turner et al., 1994). Not only was Blimp-1 mRNA induced during differentiation of BCL1 cells to an IgM secreting state, but enforced expression of Blimp-1 was sufficient to drive differentiation (Turner et al., 1994). Subsequent studies confirmed that enforced expression of Blimp-1 is sufficient to drive plasmacytic differentiation of B cells at an appropriate developmental stage (Lin et al., 2000, 2002; Piskurich et al., 2000; Schliephake and Schimpl, 1996; Shaffer et al., 2002). Furthermore, Blimp-1 is expressed in all PCs and in a small subset of germinal center B cells that have some plasma cell characteristics (Angelin-Duclos et al., 2000; Falini et al., 2000). These data are all consistent with an important role for Blimp-1 in plasma cell differentiation (reviewed in Calame, 2001; Calame et al., 2003).

Blimp-1 is a 98 kDa protein containing five zinc finger motifs, which confer sequence-specific DNA binding (Keller and Maniatis, 1992; Tunyaplin et al., 2000). The protein is a transcriptional repressor that associates with hGroucho (Ren et al., 1999) and histone deacetylases (Yu et al., 2000). Functional Blimp-1 binding sites have been identified on several direct targets including *c-myc*, *CIITA* promoter III, *Pax5*, *SpiB*, and *Id3* (Lin et

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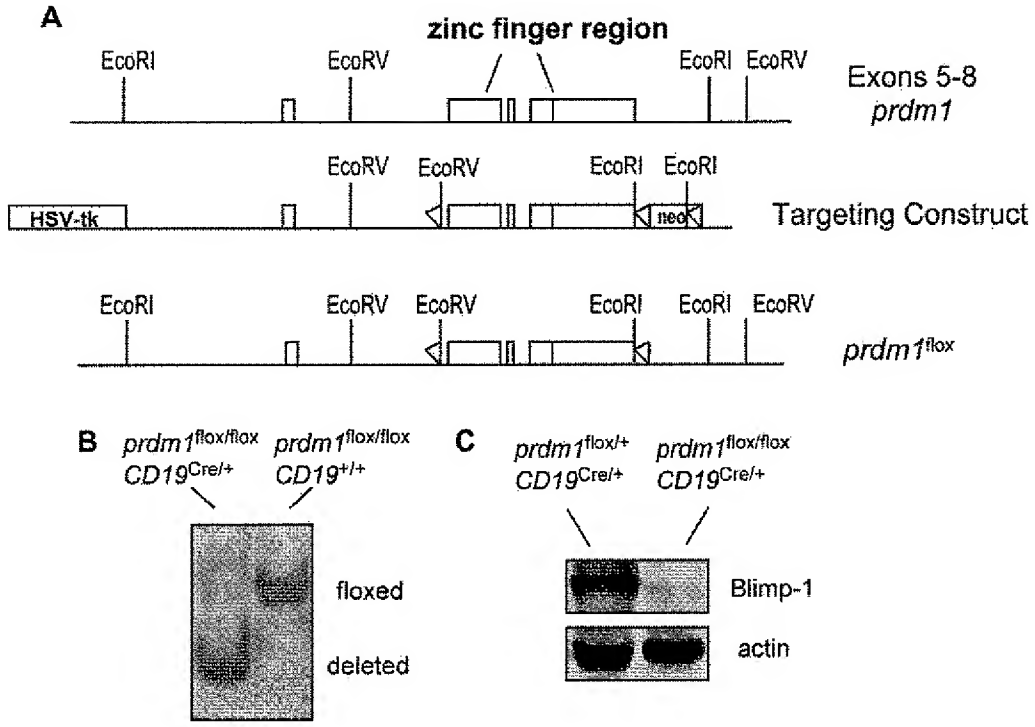


Figure 1. *Prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ Mice Have Efficient Deletion of *prdm1* in B Cells

(A) Strategy for generating an allele of *prdm1* with the zinc finger region flanked by LoxP sites (triangles). Part of the endogenous locus, targeting construct, and floxed allele after in vitro deletion of neo are shown.

(B) Southern blot analysis of B cell DNA to detect deleted and floxed *prdm1*.

(C) Immunoblots of cell lysates from splenocytes cultured in LPS for 3 days were blotted with mAb to Blimp-1.

al., 1997, 2002; Piskurich et al., 2000; Shaffer et al., 2002). In B cells, Blimp-1 regulates three large gene expression programs: cell cycle arrest, induction of Ig secretion, and inhibition of GC functions (Shaffer et al., 2002).

Although Blimp-1 is sufficient to drive plasmacytic differentiation, it is not known if Blimp-1 is required for plasmacytic differentiation. Gene targeting is the obvious way to approach this question. However, mice lacking *prdm1*, the gene encoding Blimp-1, die as early embryos (M. Davis, personal communication). This is probably because Blimp-1 is expressed in multiple embryonic cells, as early as day 7 (Chang and Calame, 2002). Therefore, we created mice in which the exons of *prdm1* that encode the zinc finger motifs of Blimp-1 are flanked by LoxP sites. Here we describe the phenotype of *prdm1*$^{flox/flox}$CD19$^{Cre/+}$ mice, which lack *prdm1* in their mature B cells. These mice have normal B cell development but cannot form PCs or secrete Ig normally. They are also defective in the formation of pre-plasma memory B cells.

Results

Conditional Deletion of *prdm1*

Exons that encode the zinc finger motifs of Blimp-1 were flanked with LoxP sites (Figure 1A). *Prdm1*$^{flox/flox}$ mice were crossed with mice expressing Cre recombinase under the control of the CD19 promoter (*CD19*$^{Cre/+}$) to achieve deletion of *prdm1* in B cells (Rickert et al., 1997). CD19 expression is B cell specific, beginning in early progenitors and continuing throughout development.

To determine the efficiency of *prdm1* deletion in B cells, B220$^+$ splenocytes were purified from *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice and DNA was analyzed by Southern blotting. There is very efficient deletion of *prdm1* in B cells of the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice (Figure 1B). Similar results were seen using *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$mice (not shown) so *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$and *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$ mice were used interchangeably. To assess Blimp-1 protein, B220$^+$ splenocytes were purified and stimulated ex vivo with LPS. Blimp-1 was almost undetectable in B cells from *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice although controls showed good expression (Figure 1C). Thus, we conservatively estimate at least 90% of the mature B cells from CD19$^{Cre/+}$*prdm1*$^{flox/flox}$ mice lack Blimp-1.

Severe Reduction in Ig Secretion and Plasma Cell Formation in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ Mice

The numbers and developmental subsets of B cells in the bone marrow and spleen of *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice appeared normal when analyzed by flow cytometry (not shown); however, serum Ig was significantly reduced in unimmunized mice (Figure 2A). To assess a TI response, *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ and littermate control mice were immunized with NP-Ficoll. Control mice showed normal increases in NP-specific serum IgM and

IgG3, but *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice had a greatly diminished serum Ig response (Figure 2B). NP-specific serum IgM, IgG1, and IgG2a were determined following immunization with NP-KLH precipitated in alum to study a TD response. The *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice showed a very large reduction in serum Ig of all 3 isotypes (Figure 2C). Failure to secrete Ig was also seen in a recall response elicited by a second immunization with NP-KLH in alum (Figure 2C).

ELISPOT assays were performed to determine if the decrease in serum Ig was due to a lack of Ig secreting cells in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice following immunization. In agreement with the decrease in serum IgM, there was a severe decrease in the number of NP-specific IgM secreting cells in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice (Figure 2B, third panel, and 2C, second panel). We also determined the numbers of CD138$^+$ B220$^{+/-}$ PCs in the spleens of the immunized mice. After immunization with NP-KLH, control mice showed increased numbers of CD138$^+$B220$^{+/-}$ PCs in a pattern consistent with an early plasma cell response and a later post-GC response (Figure 2D). However, the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice had very few CD138$^+$B220$^{+/-}$ PCs at any time (Figure 2D).

Therefore, while Blimp-1 is not required for B cell maturation, it is required for formation of CD138$^+$B220$^{+/-}$ immunoglobulin-secreting PCs both early and late in a primary immune response. It is required for production of a response to both TI and TD antigens and for serum Ig in both a primary and recall response.

Pre-Plasma Memory B Cell Formation Is Defective in a Primary Response

A six-color flow cytometric strategy, including antigen binding and cell surface phenotype (Driver et al., 2001; McHeyzer-Williams et al., 2000), was used to quantify NP$^+$ subsets following IP immunization with NP-KLH in Ribi adjuvant. Baseline labeling in spleen and bone marrow before immunization using this strategy is negligible (Figures 3A, 3C, and 3D). As antibody can be cytophilic and passively adsorb to cells spuriously conferring antigen binding (Berken and Benacerraf, 1966; Boyden, 1960), we transferred NP-immune sera (2 doses of 200 μl) into naive mice with no change in NP binding (Figures 3A, 3C, and 3D). Thus, no passively adsorbed antibody in vivo interferes with the cellular quantification using the strategy presented here.

When immunized with adjuvant alone, negligible labeling was observed (Driver et al., 2001; McHeyzer-Williams et al., 2000). At day 7, the peak of early clonal expansion (Driver et al., 2001), total NP$^+$IgD$^-$ B cells (also PI$^-$CD4$^-$CD8$^-$) were equivalent in control and *prdm1*$^{flox/flox}$CD19$^{Cre/+}$ mice (Figures 3B and 3C, first panels). In contrast, there was a 95% reduction in total CD138$^+$B220$^{+/-}$ NP$^+$ PCs in the spleen at day 7 (Figures 3B and 3C, second panels; Figure 3C; $p = 0.004$). There was a substantial reduction in NP-specific PCs on day 14 in controls but PC numbers in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice were still significantly fewer (Figure 3C; 70% reduction, $p = 0.05$) as well as total CD138$^+$ cells in spleen sections (Figure 3E). Thus, antigen-specific clonal expansion was not dependent on Blimp-1; however, consistent with previous results (Figure 2), the development of both short-lived PCs (day 7) and post-GC long-lived PCs (day 14) was blocked in the absence of Blimp-1.

In contrast to the depletion of NP$^+$ PCs, there was an increase in the frequency (Figure 3B; 31 ± 0.7% and 62 ± 6.4% controls and *prdm1*$^{flox/flox}$CD19$^{Cre/+}$ respectively, $p = 0.004$) and total numbers (Figure 3C) of CD138$^-$B220$^+$ NP$^+$ B cells that expressed GL7 (a marker for GC B cells)(Han et al., 1997) at day 7 compared to preimmune animals (negligible numbers of total NP-specific cells see Figure 3C and NP-specific GL7$^+$ cells data not shown). This trend persisted to day 14 with respect to frequency among total NP$^+$ cells (32 ± 9% and 67 ± 10% control and *prdm1*$^{flox/flox}$CD19$^{Cre/+}$, respectively, $p = 0.03$) and total numbers (Figure 3C). A similar increase in total B220$^+$GL7$^+$ cells was observed in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice from day 3 to 20 following immunization (data not shown) with larger and more numerous GCs also seen with PNA staining of spleen sections (Figure 3E). Thus, B cells lacking Blimp-1 accumulate to higher numbers in the GC compartment without differentiating into long-lived PCs.

In control animals, a third subset of NP$^+$ B cells is evident as CD138$^-$B220$^-$ (Fig3B, second panel). This memory B cell subset expresses CD79b and not GL7 (data not shown), does not secrete antibody, and has undergone affinity maturation (Driver et al., 2001). Although not found in the GC, they probably originate there during the primary response (Driver et al., 2001). These cells will be referred to as pre-plasma memory B cells or B220$^-$CD79b$^+$ cells throughout this study. The majority of NP$^+$ cells that migrate and persist in the bone marrow after priming express the CD138$^-$B220$^-$CD79b$^+$ phenotype and are also considered pre-plasma memory B cells (Figure 3D, upper panels). Thus, bone marrow migration is a reliable indicator of pre-plasma memory B cell development. In the absence of Blimp-1, pre-plasma memory B cells fail to develop fully in the spleen. Although some NP$^+$ cells have lower B220 levels in the spleen (Figure 3B, lower middle panel, 3C total numbers), there is >90% reduction in the pre-plasma memory B cell compartment in the bone marrow on day 7 and >95% reduction on day 14 (Figure 3D; day 7 $p = 0.02$, day 14 $p = 0.04$). Thus, Blimp-1 is required for the complete development of pre-plasma memory B cells.

Memory B Cells Require Blimp-1 for Further Development and Plasma Cell Differentiation

The NP$^+$ memory B cell response 5 days after antigen rechallenge was also assessed (Figure 4). Total NP-specific memory B cells were very low on day 42 before rechallenge; however, a rapid and substantial expansion of NP$^+$ B cells was seen in both control and *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (Figure 4A, first panels; Figure 4B, panel 1). In contrast, there was a profound alteration in memory B cell subsets that appeared in the absence of Blimp-1 (Figure 4A, second panels, and 4B, panels 2–4). Absence of CD138$^+$B220$^{+/-}$ NP$^+$ PCs was virtually complete with a 99% reduction in total cells (Figure 4A, second panels; Figure 4B, panel 2). Similar to the primary response, the CD138$^-$B220$^+$NP$^+$ compartment comprised the majority of the memory B cell response in the absence of Blimp-1. There were almost 10-fold more CD138$^-$B220$^+$NP$^+$ cells expressing the GC marker, GL7,

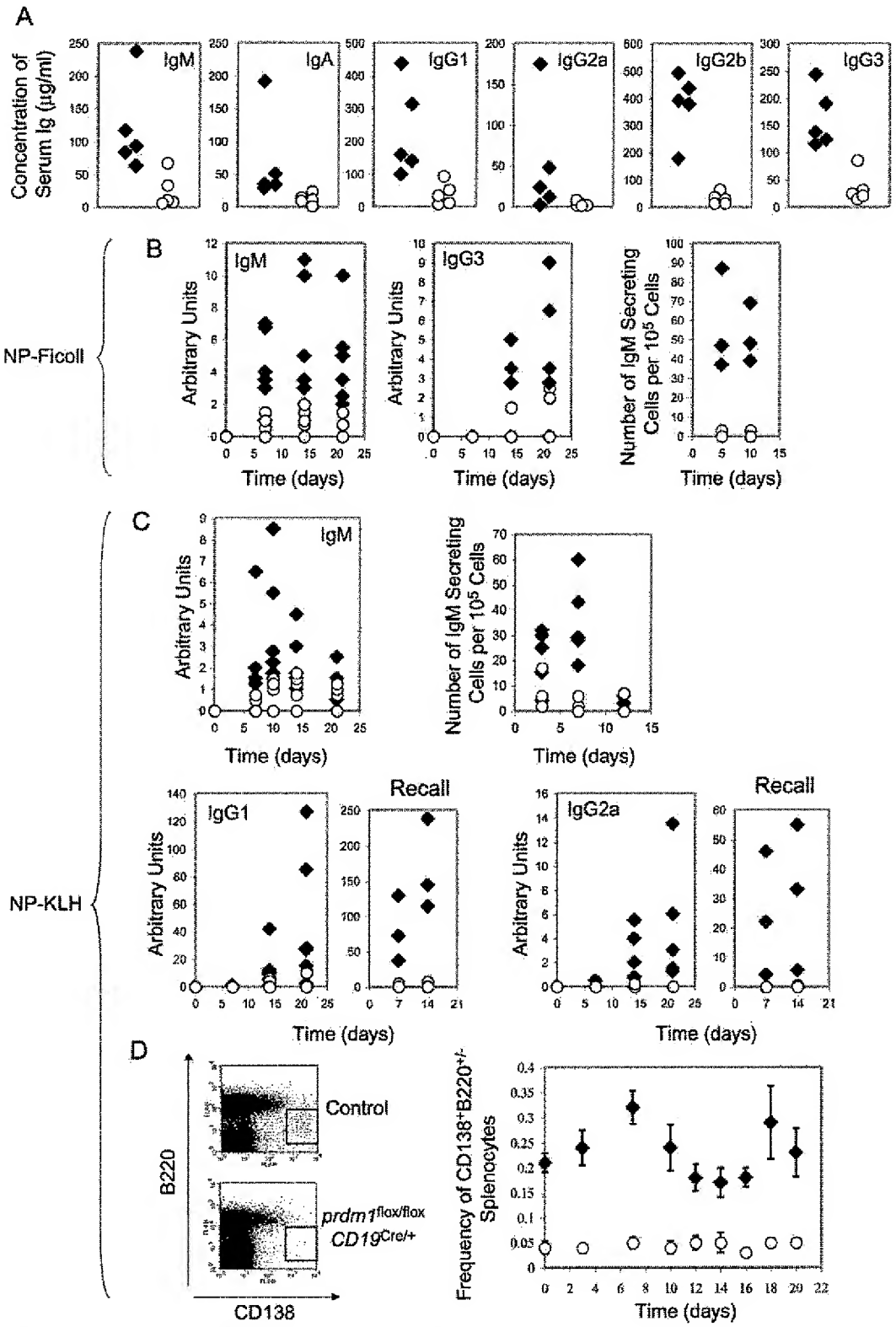


Figure 2. *Prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ Mice Do Not Develop PCs or Secrete Ig

(A) Serum from naive mice was analyzed for resting levels of IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 by ELISA. Mice were immunized with NP-Ficoll (B) or NP-KLH (C and D). Serum was analyzed by ELISA for NP-specific IgM and IgG3 (B) or NP-specific IgM, IgG1, and IgG2a (C).

in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice compared to controls (Figure 4A, third panels, Figure 4B, panel 3). Pre-plasma memory B cells (CD138$^-$B220$^-$CD79b$^+$NP$^+$) were reduced 75% in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ spleens (Figure 4B, $p = 0.04$) and reduced 98% in the bone marrow ($p = 0.006$, data not shown).

Thus, the primary GC reaction resolved 42 days after initial challenge in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. However, similar to the primary response, the NP-specific memory response accumulated aberrantly in the GL7$^+$B220$^+$NP$^+$ compartment without producing preplasma memory B cells or PCs. Thus, Blimp-1 is clearly required for normal plasma cell development at all stages of the primary and memory response.

Prdm1$^{-/-}$ B Cells Cultured Ex Vivo with LPS Proliferate, but Fail to Induce μS mRNA or Secrete Immunoglobulin

When cultured ex vivo with LPS, murine splenic B cells proliferate and differentiate into Ig-secreting cells. To assess the proliferative capacity of stimulated B cells that lack Blimp-1, *prdm1*$^{-/-}$ and control B220$^+$ cells were cultured with LPS and viable cells were counted. At all time points after LPS treatment, there were more *prdm1*$^{-/-}$ than control cells (Figure 5A). When splenocytes were labeled with CFSE, after 2 days of LPS treatment, *prdm1*$^{-/-}$ cells had lower CFSE intensity than control cells (Figure 5B), indicating that the increased number of cells in the *prdm1*$^{-/-}$ cultures was due to increased proliferation. When propidium iodide staining was performed to quantitate apoptotic cells after stimulation with LPS, no significant difference was observed in the number of apoptotic cells in *prdm1*$^{-/-}$ and control cultures (not shown).

To analyze differentiation, secreted Ig and the number of Ig secreting plasma cells was determined in the LPS cultures. Control B cells secreted large quantities of IgM and small amounts of IgG3 but *prdm1*$^{-/-}$ B cells secreted almost no IgM or IgG3 (Figure 5C). Also, there were IgM secreting cells and CD138$^+$ cells in the control cultures, but almost none in the *prdm1*$^{-/-}$ cultures (Figures 5D and 5E). Therefore, even after activation with LPS, *prdm1*$^{-/-}$ B cells are unable to differentiate into CD138$^+$ cells or secrete Ig, recapitulating ex vivo the defects observed in vivo.

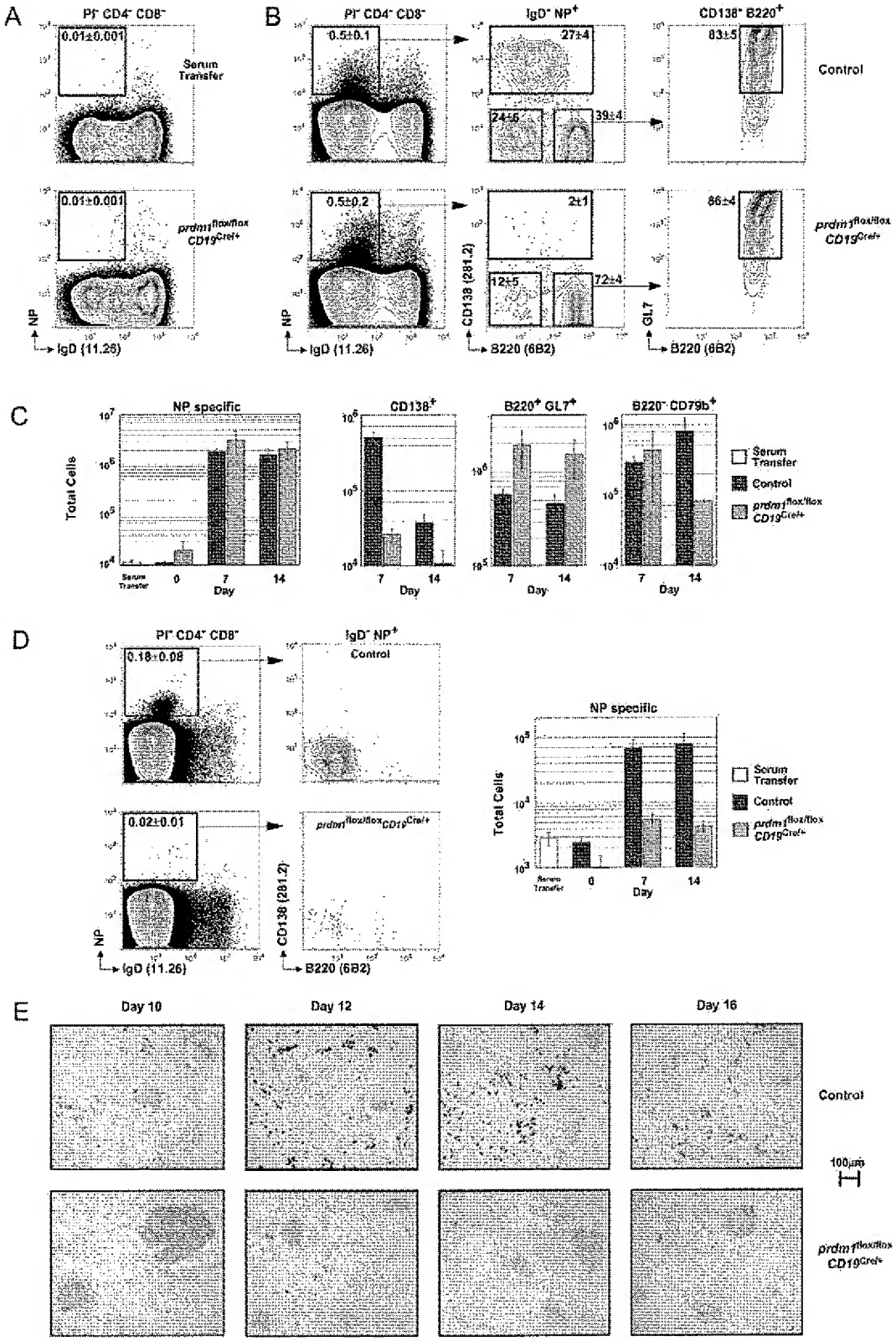
To investigate the mechanism responsible for the block in Ig secretion in *prdm1*$^{-/-}$ B cells, IgM protein in *prdm1*$^{-/-}$ and control cells was assayed by immunoblotting following culture with LPS. Cell lysates from *prdm1*$^{-/-}$ and control B cells contained similar amounts of the membrane form of μ heavy chain (μM), but control cells had more than 10-fold higher amounts of secreted μ (μS) than the *prdm1*$^{-/-}$ cells (Figure 5F). When μM and μS mRNA were analyzed by semiquantitative RT-PCR (Figure 5G), at least a 16× reduction in μS mRNA was observed in *prdm1*$^{-/-}$ B cells. We have also noted that *prdm1*$^{-/-}$ B cells are defective in induction of Ig light chain mRNA (A. Shaffer and M.S.-S., unpublished data). Thus, both the induction of Ig mRNA and the switch from μM to μS mRNA are defective in *prdm1*$^{-/-}$ B cells.

Blimp-1 and XBP-1 Have Separate Roles in Plasmacytic Differentiation

The activator XBP-1 is the only other transcription factor known to be required specifically for plasmacytic differentiation (Reimold et al., 2001). Splenic B cells lacking XBP-1 have normal levels of Blimp-1 following ex vivo stimulation, demonstrating that Blimp-1 cannot drive plasmacytic differentiation in the absence of XBP-1 and suggesting that Blimp-1 acts before XBP-1 in plasma cell development (Reimold et al., 2001). Consistent with this idea, Blimp-1 can induce XBP-1 mRNA indirectly by repressing *Pax5* (Lin et al., 2002; Shaffer et al., 2002). We analyzed XBP-1 mRNA and protein in B cells lacking Blimp-1. Semiquantitative RT-PCR showed that XBP-1 mRNA was strongly induced in LPS treated controls, but not in *prdm1*$^{-/-}$ cells (Figure 6A). Both unprocessed and processed forms of XBP-1 (Calfon et al., 2002; Iwakoshi et al., 2003; Lee et al., 2002; Yoshida et al., 2001) were induced in control cultures; however, minimal unprocessed and no processed XBP-1 was detected in *prdm1*$^{-/-}$ cultures (Figure 6B). Therefore, B cells without Blimp-1 fail to induce XBP-1 mRNA normally and are defective in their ability to make processed XBP-1 protein.

Since XBP-1 is required for plasma cell formation and because XBP-1 is not induced normally in the absence of Blimp-1, we wondered if the critical role for Blimp-1 in plasmacytic differentiation was *solely* induction of XBP-1. To investigate this possibility, LPS treated *prdm1*$^{-/-}$ splenic B cells were infected with bicistronic retroviruses expressing either Blimp-1 or XBP-1 (processed form) and YFP. YFP$^+$ cells were analyzed by flow cytometry for CD138 expression and by ELISPOT for IgM secretion. LPS treated *prdm1*$^{-/-}$ cells expressing exogenous Blimp-1 induced surface CD138 and secreted IgM, demonstrating that Blimp-1 expressed from a retrovirus can complement the *prdm1*$^{-/-}$ defect (Figures 6C and 6D). However, *prdm1*$^{-/-}$ cells infected with the XBP-1 virus were not able to induce CD138 or normally secrete IgM (Figures 6C and 6D). To confirm that functional XBP-1 was present in these cells, semiquantitative RT-PCR was performed on YFP$^+$ cells to amplify XBP-1 and DnaJ mRNA. *DnaJ* expression is activated by XBP-1 in B cells (Lee et al., 2003). In *prdm1*$^{-/-}$ cells infected with virus expressing XBP-1, and not in cells infected with control virus, XBP-1 mRNA was increased (Figure 6E). DnaJ mRNA was also induced in cells expressing XBP-1 (Figure 6E), providing evidence that the expressed XBP-1 was functional. These experiments demonstrate that Blimp-1 is fully able to rescue a defi-

Splenocytes were harvested at indicated days to quantitate NP-specific IgM secreting cells by ELISPOT (B and C). A secondary immunization with NP-KLH was given at least 6 weeks after priming and serum was analyzed by ELISA for NP-specific IgG1 and IgG2a (C). (D) On indicated days after NP-KLH, splenocytes were stained for B220 and CD138. Representative CD138 and B220 levels for day 7 post-NP-KLH are shown for *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ and control. The frequency of CD138$^+$B220$^{+/-}$ cells was determined. Each time point represents the average and SEM of at least three mice. For all panels, control mice are represented by filled diamonds and *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ by open circles.


A
PI⁻ CD4⁻ CD8⁻
0.01±0.001
Serum Transfer
0.01±0.001
prdm1flox/flox CD19Cre/+
NP
IgD (11.26)
B
PI⁻ CD4⁻ CD8⁻
0.5±0.1
IgD⁻ NP⁺
27±4
24±6
39±4
CD138⁺ B220⁺
83±5
Control
0.5±0.2
2±1
12±5
72±4
86±4
prdm1flox/flox CD19Cre/+
CD138 (281.2)
B220 (6B2)
GL7
B220 (6B2)
C
NP specific
Total Cells
Serum Transfer
0
7
14
Day
CD138⁺
B220⁺ GL7⁺
B220⁻ CD79b⁺
Serum Transfer
Control
prdm1flox/flox CD19Cre/+
D
PI⁻ CD4⁻ CD8⁻
0.18±0.08
IgD⁻ NP⁺
Control
0.02±0.01
prdm1flox/flox CD19Cre/+
NP
IgD (11.26)
CD138 (281.2)
B220 (6B2)
NP specific
Total Cells
Serum Transfer
Control
prdm1flox/flox CD19Cre/+
Day
E
Day 10
Day 12
Day 14
Day 16
Control
100μm
prdm1flox/flox CD19Cre/+

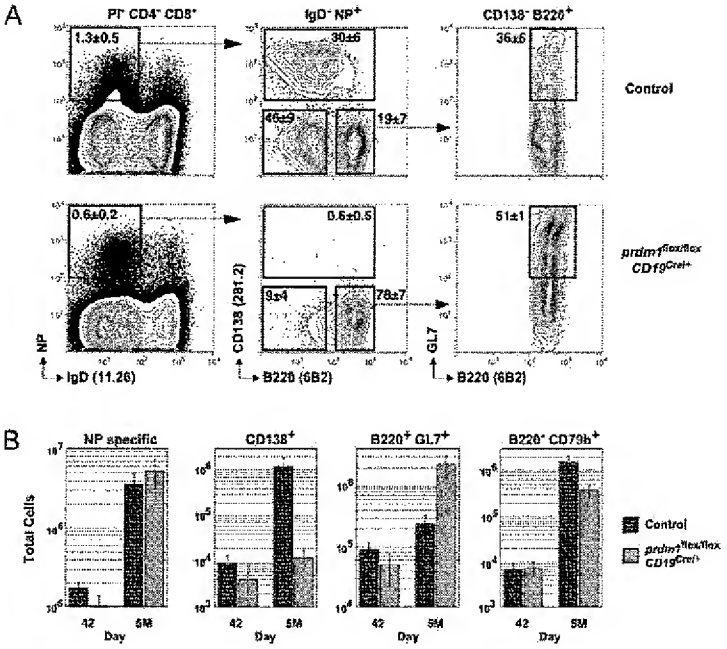


Figure 4. Memory B Cell Responses Require Blimp-1

Mice were immunized with NP-KLH in Ribi, rested for 6 weeks, and reimmunized with antigen in adjuvant. Splenocytes harvested before immunization at day 42 primary and 5 days after antigen recall, stained as described in Figure 3. (A) NP and IgD levels of $CD4^-CD8^-$ PI^- cells harvested from spleen on day 5 after recall (first two panels) with small box insert outlining NP^+IgD^- events. CD138 versus B220 levels on $PI^-CD4^-CD8^-$ NP^+IgD^- cells (second two panels). GL7 levels on $CD138^-B220^+$ NP-specific cells (third two panels), mean frequency ± SEM, n = 3. Controls displayed in upper panels and $prdm1^{flox/flox}$ $CD19^{Cre/+}$ lower panels. Mean frequency ± SEM, n = 3 across separate animals. (B) Cell numbers in spleen for each group at day 42 primary and day 5 memory: NP-specific cells ($PI^-CD4^-CD8^-NP^+IgD^-$), $CD138^+$ cells (NP-specific and $CD138^+$ $B220^{+/-}$), $B220^+$ $GL7^+$(NP-specific and $CD138^-B220^+GL7^+$), $B220^+CD79b^+$ (NP-specific and $CD138^-B220^+CD79b^+$). Mean frequencies ± SEM, n = 3.

ciency in *prdm1*, but XBP-1 cannot compensate for a lack of Blimp-1. Thus, because XBP-1 is not sufficient to drive plasmacytic differentiation in the absence of Blimp-1, Blimp-1 must have other critical targets in plasmacytic differentiation besides XBP-1.

Discussion

Requirement for Blimp-1 in Plasma Cell Formation and Ig Secretion

Blimp-1 is known to be *sufficient* to drive terminal differentiation of B cells into Ig secreting PCs (Lin et al., 2000, 2002; Piskurich et al., 2000; Schliephake and Schimpl, 1996; Shaffer et al., 2002; Turner et al., 1994). The results presented here provide unequivocal evidence that Blimp-1 is also *required* for plasmacytic differentiation and Ig secretion. This is consistent with studies where expression of a blocking form of Blimp-1 inhibited Ig secretion by B cells cultured ex vivo (Shaffer et al., 2002). However, mice expressing a blocking form of Blimp-1 did not have defective plasma cell differentiation, presumably because endogenous Blimp-1 was not fully inhibited (Angelin-Duclos et al., 2002). Although previous studies using cultured B cells treated with antisense Blimp-1 oligonucleotides suggested that Blimp-1 was required for TI, but not TD responses (Soro et al., 1999), the phenotype of $prdm1^{flox/flox}CD19^{Cre/+}$ mice establishes a role for Blimp-1 in response to both types of antigens. Mice lacking Blimp-1 in their B cells were unable to mount a normal humoral response to either a TI antigen or to the TD form (Figures 2–4). In the TD response, both early and post-germinal center responses were defective, as was a recall response to secondary challenge (Figures 2–4). However, some $prdm1^{flox/flox}CD19^{Cre/+}$ mice did have a small number of PCs and/or serum Ig following immunization. At present we cannot distinguish between the possibility that a small fraction of B cells did not delete *prdm1* or the existence of a minor, Blimp-1-indepedent pathway for PC differentiation.

Finding that $prdm1^{-/-}$ B cells are hyperproliferative (Figure 5) confirms a role for Blimp-1 in cell cycle arrest and suggests that Blimp-1 is important for limiting the number of divisions plasmablasts undergo prior to terminal differentiation. This is consistent with microarray

Figure 3. $Prdm1^{flox/flox}$ $CD19^{Cre/+}$ Mice Have Defective Development of Antigen-Specific PCs and Pre-Plasma Memory B Cells

(A) NP and IgD levels on $CD4^-CD8^-$ PI^- spleen cells before immunization. Upper panel presents a wt C57BL/6 that had received 2 × 200 μl NP-immunized serum IV before analysis and lower panel presents $prdm1^{flox/flox}$ $CD19^{Cre/+}$ animals. Inserts outline the region used for estimation of NP^+IgD^- cells with mean frequencies ± SEM across three separate animals depicted.

(B) NP and IgD levels on $CD4^-CD8^-$ PI^- cells day 7 after NP-KLH immunization (first two panels). Small box insert outlines NP^+IgD^-. CD138 versus B220 on $PI^-CD4^-CD8^-NP^+IgD^-$ cells (second two panels). GL7 levels on $CD138^-B220^+$ NP-specific cells (third two panels). Controls displayed in upper panels and $prdm1^{flox/flox}$ $CD19^{Cre/+}$ lower panels. Mean frequencies ± SEM, n = 3.

(C) Total NP-specific B cell numbers in spleen across day 0 (with serum transfer) and day 0, 7, and 14 after NP-KLH immunization (three mice/group) total NP-specific cells ($PI^-CD4^-CD8^-NP^+IgD^-$), $CD138^+$ cells (NP-specific and $CD138^-B220^{+/-}$), $B220^+$ $GL7^+$(NP-specific and $CD138^-B220^+GL7^+$), $B220^+CD79b^+$ (NP-specific and $CD138^-B220^+CD79b^+$).

(D) NP versus IgD levels on bone marrow harvested from day 7 immunized mice (first panels) mean frequencies ± SEM, n = 3 inserted. CD138 versus B220 on PI^-CD4^-NPTIgD$^-$ cells (second two panels). Total NP-specific cells in bone marrow (second panels) from two femurs mean ± SEM across three separate mice for day 0 (with serum transfer), day 0, 7, and 14 after NP-KLH immunization.

(E) Spleens from mice immunized with NP-KLH were stained for PNA (red) and CD138 (blue) 10, 12, 14, and 16 days after immunization.

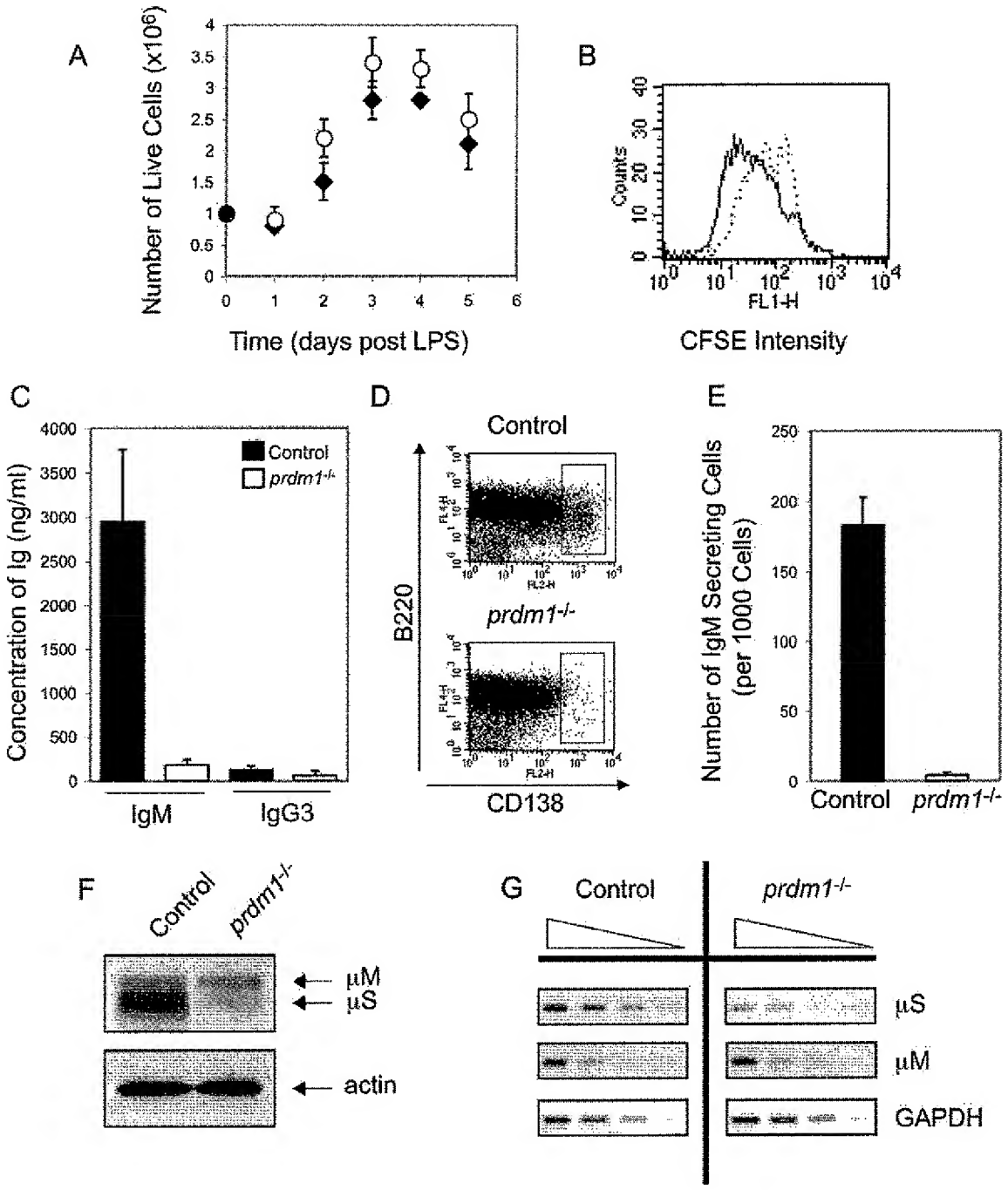


Figure 5. Upon Stimulation with LPS, *prdm1*$^{-/-}$ B Cells Proliferate but Do Not Become PCs, Secrete Ig, or Induce μS RNA or Protein

(A) Average number and SEM of viable cells during culture of B220^{+} cells with LPS (*prdm1*$^{-/-}$, open circles; control, filled diamonds). (B) Splenocytes incubated with CFSE, then cultured with LPS for 2 days, were stained with B220. The CFSE intensity of B220^{+} cells is shown (*prdm1*$^{-/-}$ B cells, solid line; control, dotted line; representative of four experiments). (C) Cell supernatants from LPS treated splenocytes were assayed for secreted IgM and IgG3 by ELISA. Splenocytes cultured in LPS for 4 days were stained for B220 and CD138 (D) or analyzed by ELISPOT to detect IgM secreting cells (E). (F) Cell lysates from splenocytes cultured for 4 days in LPS were analyzed by immunoblot to detect μM and μS protein. By day 2, cultures are at least 90% B220^{+} (data not shown). (G) Semiquantitative RT-PCR was used to detect μM and μS mRNA in LPS treated B220^{+} splenocytes.

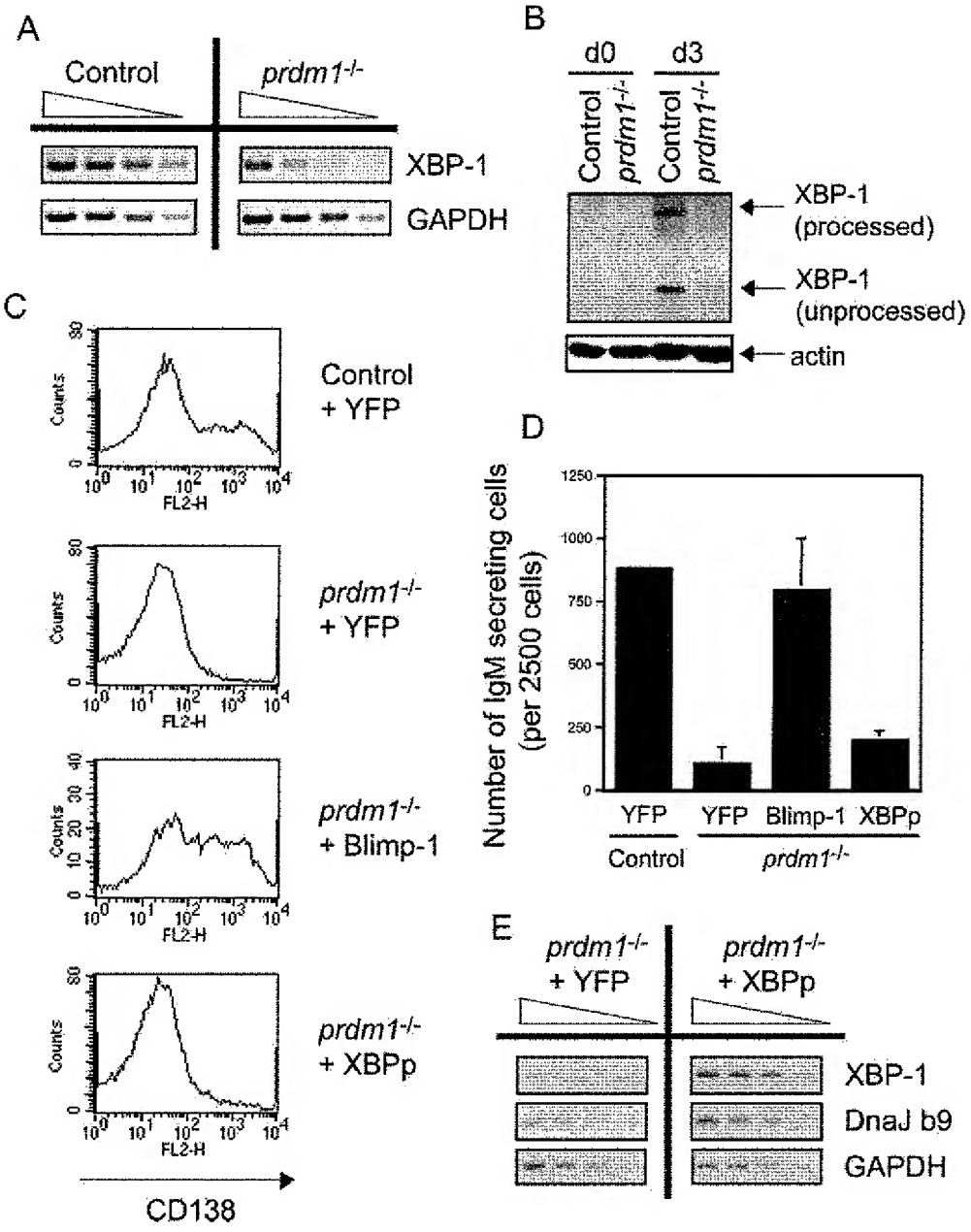


Figure 6. XBP-1 Is Not Induced or Processed in *prdm1*$^{-/-}$ B Cells but Exogenous XBP-1 Is Not Sufficient to Rescue Defective CD138 Expression and Ig Secretion in *prdm1*$^{-/-}$ B Cells

(A) RNA was harvested from B220^{+} splenocytes cultured in LPS for 4 days to be used for semiquantitative RT-PCR amplification of XBP-1 and GAPDH.

(B) Whole cell lysates from LPS treated splenocytes were immunoblotted to detect XBP-1 protein.

(C, D, and E) LPS treated splenocytes were infected with a retrovirus expressing YFP, YFP and Blimp-1, or YFP and XBP-1p. After 3 more days of culture, B220^{+}YFP^{+} cells were analyzed for CD138 expression (C) and sorted for ELISPOT analysis to detect the number of IgM secreting cells (D). RNA was also extracted from YFP^{+} cells for semiquantitative RT-PCR detection of XBP-1, DnaJ b9, and GAPDH (E).

studies that identified a large Blimp-1-dependent program that represses proliferation (Shaffer et al., 2002) and with the fact that terminally differentiated PCs do not divide. The Blimp-1-regulated proliferation program includes repression of c-*myc*, *E2F-1*, and other genes necessary for cell division as well as induction of cdk inhibitors p21 and p18 (Lin et al., 1997, 2000; Shaffer et al., 2002). Induction of p18 is particularly important, since mice lacking p18 have a severe reduction in antibody-containing plasmacytoid cells (Tourigny et al.,

2002). It is also consistent with hyperproliferation of B cells expressing a blocking form of Blimp-1 (Angelin-Duclos et al., 2002). However, proliferation of *prdm1*$^{-/-}$ B cells does stop after 3 or 4 days of ex vivo culture and we have not yet observed B cell lymphomas in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. Thus, there appear to be Blimp-1-independent, as well as Blimp-1-dependent mechanisms for regulating proliferation of maturing B cells.

Multiple mechanisms could be responsible for the defect in Ig secretion in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. Previous studies (Lin et al., 2002) showed that Blimp-1 represses *Pax5*, relieving Pax5-dependent repression of J chain, Ig, and XBP-1 transcription (Shaffer et al., 1997; Singh and Birshtein, 1993; Wallin et al., 1999). The *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice confirm Blimp-1-dependent induction of Ig (Figure 5; data not shown) and XBP-1 mRNA (Figure 6). B cells lacking Blimp-1 also failed to generate μS mRNA and protein (Figure 5). This suggests a previously unknown requirement for Blimp-1 in the developmentally regulated switch from membrane (M) to secreted (S) μ. Whether this switch depends on differential polyA site usage, splicing, or mRNA stability remains unresolved (Berberich and Schimpl, 1990; Peterson and Perry, 1989; Phillips et al., 2001; Yan et al., 1995) and it will be interesting to determine if Blimp-1 affects the activity of Cstf64 polyadenylation complexes (Takagaki and Manley, 1998) or factors such as U1A (Phillips et al., 2001) that affect μ mRNA processing and stability. Alternatively, Blimp-1 could be required for an event developmentally upstream of the μM to μS switch. Future studies using inducible Cre recombinase may be useful in addressing this issue.

The phenotype of the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice establishes that Blimp-1 is required to drive the major portion of plasmacytic differentiation and that timely arrest of cell cycle and induction of Ig secretion, two critical components of this process, depend on Blimp-1. It rules out the existence of any other transcriptional regulator with functions that can replace those of Blimp-1 in this context.

Blimp-1 and XBP-1

XBP-1 is the only transcription factor other than Blimp-1 known to be uniquely required for plasma cell formation and Ig secretion (Reimold et al., 2001). Since B cells lacking XBP-1 express Blimp-1 normally (Reimold et al., 2001), Blimp-1 is not sufficient for plasmacytic differentiation without XBP-1. The defect in XBP-1 mRNA induction observed in *prdm1*$^{-/-}$ B cells (Figure 6) is consistent with Blimp-1 acting before XBP-1 and confirms earlier data (Lin et al., 2002; Shaffer et al., 2002) showing Blimp-1 induces XBP-1 mRNA. XBP-1 is also subject to posttranscriptional regulation. The unfolded protein response (UPR) activates IRE1 endonuclease-dependent processing of XBP-1 mRNA to generate mRNA encoding a more active and stable form of XBP-1 (Calfon et al., 2002; Lee et al., 2002; Yoshida et al., 2001), which is required for PC differentiation (Iwakoshi et al., 2003). Furthermore, Ig synthesis is necessary for the B cell UPR that activates XBP-1 mRNA processing (Iwakoshi et al., 2003). Since Blimp-1 is required for induction of μS mRNA and protein (Figure 5), the induction of Ig synthesis and subsequent UPR-dependent processing of XBP-1 mRNA is a second, indirect, way that Blimp-1 regulates XBP-1.

Accordingly, we asked if failure to induce and process XBP-1 mRNA was the sole reason *prdm1*$^{-/-}$ B cells were blocked for PC differentiation. Forced expression of XBP-1 (processed form) was unable to restore differentiation and Ig secretion to *prdm1*$^{-/-}$ B cells following LPS treatment (Figure 6), demonstrating that Blimp-1 is required to regulate genes in addition to XBP-1. Thus, although Blimp-1 is important for regulation of XBP-1, Blimp-1 and XBP-1 each regulate unique targets. For example, Blimp-1 suppresses proliferation whereas proliferation is normal in XBP-1$^{-/-}$ B cells (Reimold et al., 2001). Furthermore, Blimp-1 represses genes involved in germinal center B cell functions (Shaffer et al., 2002) and *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice have abnormally large germinal centers (Figures 3 and 4). However, in XBP-1$^{-/-}$ chimeras, germinal centers are normal (Reimold et al., 2001). Finally, μS mRNA levels are normal in XBP-1$^{-/-}$ B cells (Reimold et al., 2001). Therefore these functions may be specifically dependent on Blimp-1. With respect to XBP-1, few targets have been identified, but *Grp78* (Yoshida et al., 2001) and *DnaJ* (Lee et al., 2003) may be unique direct targets of XBP-1.

A Role for Blimp-1 in Post-GC Memory Cells

Primary exposure to antigen drives the development of PCs and long-lived, antigen-experienced memory cells (MacLennan and Gray, 1986; McHeyzer-Williams, 2003; McHeyzer-Williams and Ahmed, 1999). Upon resolving the cellular basis of the plasma cell defect in *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice, we revealed an unexpected role for Blimp-1 in B cell memory (Figures 3 and 4).

The cellular organization of immune memory in vivo remains poorly understood. T_H memory cells exist in at least two forms, referred to as "central" and "effector," based on recirculation patterns, functional plasticity, and the rapidity of response to antigen recall (Lanzavecchia and Sallusto, 2002). B cell memory may also exist within these functional divisions (McHeyzer-Williams, 2003). CD138^{-}B220^{+} memory B cells recirculate through spleen and peripheral LNs (McHeyzer-Williams et al., 2000), have greater proliferative capacity on adoptive transfer, but produce fewer PCs as expected of a central memory compartment. In contrast, CD79b^{+}B220^{-} pre-plasma memory B cells appear mainly in the bone marrow (McHeyzer-Williams et al., 2000), differentiate rapidly into PCs on antigen recall (McHeyzer-Williams et al., 2000), and may be capable of homeostatic conversion to PCs in the absence of antigen (Bernasconi et al., 2002; O'Connor et al., 2002). However, they are not PCs, do not secrete antibody spontaneously (McHeyzer-Williams et al., 2000), and do exhibit attributes of an effector memory compartment. Our data show that Blimp-1 is required for the complete development of the pre-plasma memory B cell compartment (Figures 3 and 4). While other models are not ruled out, the simplest explanation of the data is a linear progression of post-germinal center development as outlined in Figure 7.

In this model, the GC reaction in a primary response produces B220^{+} central memory B cells. These cells appear rapidly in the marginal zones of the spleen (Liu

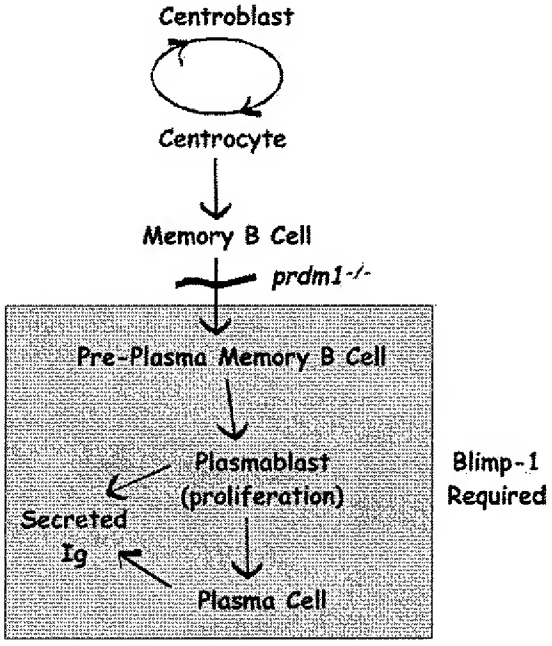


Figure 7. Model for Post GC B Cell Development
The gray box indicates stages that require Blimp-1.

et al., 1988), where they may encounter residual antigen and accessory signals (Balazs et al., 2002) to develop into pre-plasma memory B cells and/or PCs. These populations have a propensity to home to the bone marrow (Driver et al., 2001; McHeyzer-Williams et al., 2000) and persist in the absence of further antigen exposure (McHeyzer-Williams et al., 2000). Differentiation to PCs may also occur in the marrow (O'Connor et al., 2002). Localization and control of these changes remain speculative, but identifying a role for Blimp-1 provides new insight into their relationship and the basis for further experimentation.

It is not clear if $B220^+$ NP^+ B cells that persist in the absence of Blimp-1 are fully developed. They bind equivalent levels of NP-APC under saturating conditions (Figure 4; Total NP^+ B cells MFI 220 ± 13 and 310 ± 12; $B220^+$ NP^+ B cells MFI 280 ± 20 and 290 ± 16), suggesting expression of similar affinity BCR in control and *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice (McHeyzer-Williams et al., 2000). Without the capacity to produce antibody, it is difficult to test the function of Blimp-1-deficient B cells as bona fide memory cells on adoptive transfer (McHeyzer-Williams et al., 2000). It will also be important to evaluate the mutational status of the memory responders in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice to assess more directly the activity of the primary GC reaction. Blimp-1-deficient B cells underwent rapid clonal expansion but then exhibited the same block in pre-plasma memory B cell and PC development as in the primary response (Figure 4). Interestingly, the selective pressures exerted by antigen rechallenge revealed even fewer residual PCs than in the primary response. The exaggerated levels of $GL7^+$ NP-specific cells at day 5 suggest extensive secondary GC formation that is an abnormal response to antigen recall (MacLennan and Gray, 1986). These aberrant memory responses may be manifestations of the same defect exhibited in the primary response, or they may indicate incomplete development of the $B220^+$ central memory compartment itself. In either case, an effective memory B cell response to antigen recall cannot be mounted in the absence of Blimp-1. Thus, in recall as well as primary responses, Blimp-1 is critical for memory and PC development.

Experimental Procedures

Generation of *prdm1*$^{Flox/Flox}$*CD19*$^{Cre/+}$ Mice

Exons 6-8 of *prdm1* (with 5′ LoxP site introduced by site-directed mutagenesis), 8 kb of 5′ sequence, 1 kb of 3′ sequence, a neomycin resistance gene (neo) flanked by LoxP sites, and an HSV-tk gene were inserted into pKS after replacing its multiple cloning site (Figure 1). R1 embryonic stem (ES) cells were electroporated and cultured using purified LIF (Hadjantonakis et al., 1999; Mereau et al., 1993; Nagy et al., 1993). Fialuridine and neomycin were used for negative and positive selection. Colonies were screened by PCR and Southern analysis for homologous recombination. Positive clones were injected into C57/BL6 blastocysts. Chimera progeny were crossed with mice expressing Cre ubiquitously to generate *prdm1*$^{+/-}$ mice (Lewandoski et al., 1997). Targeted ES clones were also transfected with a Cre vector and screened for deletion of neo. Appropriate clones were injected into C57/BL6 blastocysts. Chimera progeny were crossed to each other, to *prdm1*$^{+/-}$ mice, and to *CD19*$^{Cre/+}$ mice (Rickert et al., 1997) to generate *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$ and *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. *Prdm1*$^{flox/-}$*CD19*$^{+/+}$, *prdm1*$^{flox/flox}$*CD19*$^{+/+}$, *prdm1*$^{flox/+}$*CD19*$^{Cre/+}$, and *prdm1*$^{-/+}$*CD19*$^{Cre/+}$ were used as littermate controls.

Southern Blots

Genomic DNA was digested with EcoRI and hybridized with a 1 kb KpnI/EcoRV fragment of *prdm1* upstream of the deleted exons to detect endogenous, floxed, and deleted *prdm1* as 15, 13.5, and 10 kb bands respectively. The Cre recombinase gene was detected by BamHI digestion and probing with Cre sequence to reveal 6.5 and 2.5kb bands. To determine the deletion efficiency of *prdm1*, DNA from purified $B220^+$ splenocytes of *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$, *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$, and control mice was analyzed.

Immunization

Prdm1$^{flox/flox}$*CD19*$^{Cre/+}$ and littermate control mice, 7- to 12-week-old, were immunized intraperitoneally (IP) with either 25 μg of (4-hydroxy-3-nitrophenyl)acetyl (NP)-Ficoll in 0.1 ml of PBS or 100 μg of NP-keyhole limpet hemocyanin (KLH) alum precipitated. For a recall response, the same dose of NP-KLH was given at least 6 weeks after the initial dose. For studies on NP-specific B cells, mice were immunized IP with 400 μg NP-KLH in Ribi adjuvant (Corixa). For the memory response, 6 weeks after primary immunization mice were reimmunized with 400 μg NP-KLH in Ribi adjuvant. Serum transfer experiments used pooled immune serum from NP-immune mice (3 × day 14, 3 × day 42, 5 × day 5 memory) with 200 μl injected into the tail vein of naive C57BL/6 mice, 3 days and 24 hr before analysis.

B Cell Culture

Splenocytes were suspended in NH_4Cl buffer (Sigma) to lyse red blood cells and plated (10^6 cells/ml) in RPMI with 10% FCS, β-mercaptoethanol, and gentamicin. Stimulation was with 10 μg/ml of lipopolysaccharide (LPS) (Sigma).

B Cell Purification

Freshly harvested splenocytes were incubated with APC conjugated α-mouse B220 (BD Pharmingen) in PBS with 1% BSA, 2 mM EDTA, and 2% FCS, washed, and incubated with goat α-rat IgG1 microbeads from Miltenyi. $B220^+$ cells were purified by positive selection using a MACS column (Miltenyi) according to manufacturer's instructions. Purity was assessed by flow cytometry.

Western Blots

Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described (Lin et al., 2000). Antibodies were monoclonal α-mouse Blimp-1 (Chang and Calame, 2002), monoclonal α-mouse β-actin (Sigma), goat α-mouse IgM (Roche), polyclonal rabbit α-mouse XBP-1 (Santa Cruz Biotechnology), goat α-mouse IgG1 conjugated to peroxidase (Roche), and α-goat IgG conjugated to peroxidase (Roche). For μS and μM blots, whole cell lysates were not centrifuged before SDS-PAGE. For XBP-1 blots, splenocytes were stimulated with 25 μg/ml LPS and, for 1 hr before lysis in 2×SDS loading buffer, treated with MG132 (10 μM, Sigma).

ELISA

To detect total IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in unimmunized mice, NP-specific IgM, IgG1, IgG2a, and IgG3 in immunized mice (6 control and 6 *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ for primary response and 3 of each for recall) and secreted IgM and IgG3 in the cell supernatants of LPS stimulated splenocytes (3 days), enzyme-linked immunosorbent assays (ELISA) were performed as previously described (Angelin-Duclos et al., 2002). Additionally, for NP-specific Ig detection, plates were coated with NP(25)-BSA (Biosearch Technology) and for total IgA, with anti-IgA (BD Pharmingen).

ELISPOT

Enzyme-linked immunospot (ELISPOT) assays were performed (Angelin-Duclos et al., 2002) to detect NP-specific IgM secreting splenocytes from immunized mice and IgM secreting splenocytes after 4 days of LPS.

Flow Cytometry

For non-NP-specific experiments, splenocytes were prepared or cultured as above and stained in PBS with 1% BSA, 2 mM EDTA, 2% FCS, and 0.03% NaN_3 on ice for 45 min using APC α-mouse B220, FITC α-mouse B220, and PE α-mouse CD138 (all from BD Pharmingen). For analysis of NP$^+$ B cells, spleen and bone marrow were harvested as described (McHeyzer-Williams et al., 2000). Cells were stained at 2×10^6 cells/ml on ice for 45 min. The following antibodies were used for labeling cells and conjugated in the McHeyzer-Williams laboratory unless otherwise noted: Cy5PE-H129.19 (α-CD4; BD Pharmingen), Cy5PE-53-6.7 (α-CD8; BD Pharmingen), FITC-11.26 (α-IgD; a gift from F. Finkelman, Univ. Cincinnati Medical Center, Cincinnati, OH), biotin-11.26, allophycocyanin (APC)-conjugated NP (4-hydroxy-3-nitrophenyl), PE-281-2 (α-CD138; BD Pharmingen), Cy7PE-6B2 (α-B220, Caltag), FITC-GL7 (BD Pharmingen), biotin-HM79b (α-Igβ / CD79b), and streptavidin-Cy7APC (BD Pharmingen) as a second step revealing reagent. Cells were washed twice in PBS with 5% FCS and resuspended in 2 μg/ml propidium iodide (PI) with 5% FCS. Samples were analyzed using Cell Quest software on a FACS Vantage SE (BD Pharmingen). Standard analog compensation was used with Ominicomp correction between Cy5PE, Cy7PE, and APC, Cy7APC. Data were analyzed using FlowJo software (Tree Star). Profiles are presented as 5% probability contours with outliers.

CFSE Analysis

Splenic B cells prepared as above were incubated at 37°C for 15 min in 1 μM CFSE (Molecular Probes) in PBS. Cells were washed with media and plated. On multiple days after staining, cells were stained for B220 and analyzed by flow cytometry.

Semiquantitative RT-PCR

B220$^+$ cells cultured for 4 days with LPS or YFP$^+$ cells after retroviral infection (see below) were harvested and suspended in Trizol (Invitrogen Life Technologies). RNA was purified according to the manufacturer's instructions. cDNA was generated with AMV reverse-transcriptase (Invitrogen Life Technologies) according to the manufacturer instructions and PCR was performed on 4-fold dilutions of the cDNA using primers for XBP-1 and GAPDH (Shaffer et al., 2002), μS (5′-TCTGCCTTCACCACAGAAG-3′ and 5′-TAGCATG GTCAATAGCAGG-3′), μM (5′-GGCTTTGAGAACCTGTGGA-3′ and 5′-TTACAGCTCAGCTGTCTGT-3′), and DnaJ b9 (5′-AACACTCGGT CTAAGAAGC-3′ and 5′-ATCAGTGTATGTAGTAACC-3′).

Plasmids

Retroviral vectors pGC-YFP and pGC-Blimp-1-YFP have been described (Piskurich et al., 2000). pGC-XBPp-YFP was generated by the blunt ligation of XBP-1 cDNA from a plasmid that produces only processed XBP-1 protein, pFLAG.XBP1p.CMV2, into the pGC-YFP vector (Calfon et al., 2002).

Retroviral Transduction

The procedure for retrovirus vectors can be found at http://www.stanford.edu/group/nolan/index.html. For pseudotyped virus, 15 μg of retroviral vector, 15 μg of pSV-ψ$^-$-E-MLV, and 15 μg of VSV-G (pMD.G) was transfected into Phoenix cells. Viral supernatants were concentrated as described (Piskurich et al., 2000). Splenic cells treated with LPS (25 μg/ml) overnight were infected with concentrated virus stock (m.o.i. of 2–5). Three days after infection, cells were analyzed by FACS. YFP$^+$ cells were sorted by flow cytometry and used for ELISPOT analysis and semiquantitative RT-PCR.

Immunohistochemistry

Spleens were processed and stained as described (Angelin-Duclos et al., 2000) using the biotin-avidin-peroxidase system to detect PNA and the alkaline phosphatase system to detect CD138.

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